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(54) Title: ANTIBODIES AND IMMUNOGLOBULIN FUSION PROTEINS HAVING MODIFIED EFFECTOR FUNCTIONS AND USES THEREFOR		
(57) Abstract <p>CTLA4-immunoglobulin fusion proteins having modified immunoglobulin constant region-mediated effector functions, and nucleic acids encoding the fusion proteins, are described. The CTLA4-immunoglobulin fusion proteins comprise two components: a first peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region which is modified to reduce at least one constant region-mediated biological effector function relative to a CTLA4-IgG1 fusion protein. The nucleic acids of the invention can be integrated into various expression vectors, which in turn can direct the synthesis of the corresponding proteins in a variety of hosts, particularly eukaryotic cells. The CTLA4-immunoglobulin fusion proteins described herein can be administered to a subject to inhibit an interaction between a CTLA4 ligand (e.g., B7-1 and/or B7-2) on an antigen presenting cell and a receptor for the CTLA4 ligand (e.g., CD28 and/or CTLA4) on the surface of T cells to thereby suppress an immune response in the subject, for example to inhibit transplantation rejection, graft versus host disease or autoimmune responses.</p>		

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ANTIBODIES AND IMMUNOGLOBULIN FUSION PROTEINS HAVING MODIFIED EFFECTOR FUNCTIONS AND USES THEREFOR

Background of the Invention

5 While antibody variable regions interact with foreign antigens, antibody constant regions mediate antibody effector functions. More specifically, the Fc portion of antibodies contain the majority of the surfaces that define the effector functions on immunoglobulins (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 283-314). One exemplary effector function is complement activation. In order
10 to active complement by the classical pathway, the Fc must possess appropriate amino acids to bind C1q. Moreover, the hinge region must allow the Fab arms to move so that there is appropriate access to the site. Another antibody effector function involves the targeting of bound foreign antigens to phagocytes via Fc receptors. Different classes of antibodies differ in their effector functions, and mutations to key regions of antibody
15 molecules can result in changes in antibody effector function.

To induce antigen-specific T cell activation and clonal expansion, two signals provided by antigen-presenting cells (APCs) must be delivered to the surface of resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165:302-319; Mueller, D.L., et al. (1990) *J. Immunol.* 144:3701-3709; Williams, I.R. and Unanue, E.R. (1990) *J. Immunol.* 145:85-93). The first signal, which confers specificity to the immune response, is mediated via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Schwartz, R.H. (1990) *Science* 248:1349-1356). Costimulation
25 is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. (1988) *J. Immunol.* 140:3324-3330; Linsley, P.S., et al. (1991) *J. Exp. Med.* 173:721-730; Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci. USA.* 88:6575-6579; Young, J.W., et al. (1992) *J. Clin. Invest.* 90:229-237; Koulova, L., et al. (1991) *J. Exp. Med.* 173:759-762; Reiser, H., et al. (1992) *Proc. Natl. Acad. Sci. USA.* 89:271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* 144:4579-4586; LaSalle, J.M., et al., (1991) *J. Immunol.* 147:774-80; Dustin, M.I., et al., (1989) *J. Exp. Med.* 169:503; Armitage, R.J., et al. (1992) *Nature* 357:80-82; Liu, Y., et al. (1992) *J. Exp. Med.* 175:437-445).

35 Considerable evidence suggests that the B7-1 protein (CD80; originally termed B7), expressed on APCs, is one such critical costimulatory molecule (Linsley, P.S., et al., (1991) *J. Exp. Med.* 173:721-730; Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci. USA.* 88:6575-6579; Koulova, L., et al., (1991) *J. Exp. Med.* 173:759-762; Reiser, H., et

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- al. (1992) *Proc. Natl. Acad. Sci. USA* 89:271-275; Linsley, P.S. et al. (1990) *Proc. Natl. Acad. Sci. USA* 87: 5031-5035; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174:625-631.). Recent evidence suggests the presence of additional costimulatory molecules on the surface of activated B lymphocytes (Boussiotis V.A., et al. (1993) *Proc. Natl. Acad. Sci. USA*, 90:11059-11063; Freeman G.J., et al. (1993) *Science* 262:907-909; Freeman G.J., et al. (1993) *Science* 262:909-911; and Hathcock K.S., et al. (1993) *Science* 262:905-907). The human B lymphocyte antigen B7-2 (CD86) has been cloned and is expressed by human B cells at about 24 hours following stimulation with either anti-immunoglobulin or anti-MHC class II monoclonal antibody (Freeman G.J., et al. (1993) *Science* 262:909-911). At about 48 to 72 hours post activation, human B cells express both B7-1 and a third CTLA4 counter-receptor which is identified by a monoclonal antibody BB-1, which also binds B7-1 (Yokochi, T., et al. (1982) *J. Immunol.* 128:823-827). The BB-1 antigen is also expressed on B7-1 negative activated B cells and can costimulate T cell proliferation without detectable IL-2 production, indicating that the B7-1 and BB-1 molecules are distinct (Boussiotis V.A., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:11059-11063). The presence of these costimulatory molecules on the surface of activated B lymphocytes indicates that T cell costimulation is regulated, in part, by the temporal expression of these molecules following B cell activation.
- B7-1 is a counter-receptor for two ligands expressed on T lymphocytes. The first ligand, termed CD28, is constitutively expressed on resting T cells and increases after activation. After signaling through the T cell receptor, ligation of CD28 induces T cells to proliferate and secrete IL-2 (Linsley, P.S., et al. (1991) *J. Exp. Med.* 173: 721-730; Gimmi, C.D., et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:6575-6579; Thompson, C.B., et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:1333-1337; June, C.H., et al. (1990) *Immunol. Today* 11:211-6; Harding, F.A., et al. (1992) *Nature* 356:607-609.). The second ligand, termed CTLA4, is homologous to CD28, but is not expressed on resting T cells and appears following T cell activation (Brunet, J.F., et al., (1987) *Nature* 328:267-270). Like B7-1, B7-2 is a counter-receptor for both CD28 and CTLA4 (Freeman G.J., et al. (1993) *Science* 262:909-911). CTLA4 was first identified as a mouse cDNA clone, in a library of cDNA from a cytotoxic T cell clone subtracted with RNA from a B cell lymphoma (Brunet, J.F., et al. (1987) *supra*). The mouse CTLA4 cDNA was then used as a probe to identify the human and mouse CTLA4 genes (Harper, K., et al. (1991) *J. Immunol.* 147:1037-1044; and Dariavich, et al. (1988) *Eur. J. Immunol.* 18(12):1901-1905, sequence modified by Linsley, P.S., et al. (1991) *J. Exp. Med.* 174:561-569). A probe from the V domain of the human gene was used to detect

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the human cDNA which allowed the identification of the CTLA4 leader sequence (Harper, K., et al. (1991) *supra*).

Soluble derivatives of cell surface glycoproteins in the immunoglobulin gene superfamily have been made consisting of an extracellular domain of the cell surface glycoprotein fused to an immunoglobulin constant (Fc) region (see e.g., Capon, D.J. *et al.* (1989) *Nature* 337:525-531 and Capon U.S. Patents 5,116,964 and 5,428,130 [CD4-IgG1 constructs]; Linsley, P.S. *et al.* (1991) *J. Exp. Med.* 173:721-730 [a CD28-IgG1 construct and a B7-1-IgG1 construct]; and Linsley, P.S. *et al.* (1991) *J. Exp. Med.* 174:561-569 and U.S. Patent 5,434,131 [a CTLA4-IgG1]). Such fusion proteins have proven useful for studying receptor-ligand interactions. For example, a CTLA4-IgG immunoglobulin fusion protein was used to study interactions between CTLA4 and its natural ligands (Linsley, P.S., et al., (1991) *J. Exp. Med.* 174:561-569; International Application WO93/00431; and Freeman G.J., et al. (1993) *Science* 262:909-911).

The importance of the B7:CD28/CTLA4 costimulatory pathway has been demonstrated *in vitro* and in several *in vivo* model systems. Blockade of this costimulatory pathway results in the development of antigen specific tolerance in murine and human systems (Harding, F.A., et al. (1992) *Nature* 356:607-609; Lenschow, D.J., et al. (1992) *Science* 257:789-792; Turka, L.A., et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:11102-11105; Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6586-6590; Boussiotis, V., et al. (1993) *J. Exp. Med.* 178:1753-1763). Conversely, transfection of a B7-1 gene into B7-1 negative murine tumor cells to thereby express B7-1 protein on the tumor cell surface induces T-cell mediated specific immunity accompanied by tumor rejection and long lasting protection to tumor challenge (Chen, L., et al. (1992) *Cell* 71:1093-1102; Townsend, S.E. and Allison, J.P. (1993) *Science* 259:368-370; Baskar, S., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5687-5690.). Therefore, approaches which manipulate the B7:CD28/CTLA4 interaction to thereby stimulate or suppress immune responses would be beneficial therapeutically.

Summary of the Invention

One embodiment this invention pertains to modified antibody proteins which have been mutated to alter more than one antibody effector function. In another aspect, the invention pertains to the nucleic acids which encode the mutated antibody molecules. In a preferred embodiment, the antibody molecules of the present invention have been mutated to reduce both complement activation and Fc receptor binding. Any class of antibody can be used to practice the present invention.

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In one embodiment this invention pertains to immunoglobulin fusion proteins having modified immunoglobulin constant (IgC) region-mediated effector functions and to nucleic acids encoding the proteins. In a preferred embodiment the immunoglobulin fusion protein is a CTLA4-immunoglobulin fusion protein. In one embodiment, the fusion proteins of the present invention have been constructed by fusing a peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region to create a CTLA4Ig fusion protein. In another embodiment, the variable regions of immunoglobulin heavy and light chains have been replaced by the B7-binding extracellular domain of CTLA4 to create CTLA4-Ab fusion proteins. As used herein, the term "CTLA4-immunoglobulin fusion protein" refers to both the CTLA4Ig and CTLA4-Ab forms. In a preferred embodiment, the fusion proteins of the invention have been modified to reduce their ability to activate complement and/or bind to Fc receptors. In one embodiment, an IgC region of an isotype other than Cγ1 is used in the fusion protein and the modified effector function(s) can be assessed relative to a Cγ1-containing molecule (e.g., an IgG1 fusion protein). In another embodiment, a mutated IgC region (of any isotype) is used in the fusion protein and the modified effector function can be assessed relative to an antibody or Ig fusion protein containing the non-mutated form of the IgC region.

The mutated antibodies or fusion proteins of the invention are useful in any setting in which it is desirable to limit multiple antibody effector functions. The CTLA4-immunoglobulin fusion proteins of the invention are useful for inhibiting the interaction of CTLA4 ligands (e.g., B7 family members such as B7-1 and B7-2) with receptors on T cells (e.g., CD28 and/or CTLA4) to thereby inhibit delivery of a costimulatory signal in the T cells and thus downmodulate an immune response. Use of the mutated antibodies or fusion proteins of the invention, such as CTLA4-immunoglobulin fusion proteins is applicable in a variety of situations, such as to inhibit transplant rejection or autoimmune reactions in a subject. In these situations, immunoglobulin constant region-mediated biological effector mechanisms, such as complement-mediated cell lysis, Fc receptor-mediated phagocytosis or antibody-dependent cellular cytotoxicity, may induce detrimental side effects in the subject and are therefore undesirable. The CTLA4-immunoglobulin fusion proteins of the invention exhibit reduced IgC region-mediated effector functions compared to a CTLA4-immunoglobulin fusion protein in which the IgG1 constant region is used and, thus are likely to have improved immunoinhibitory properties. These compositions can also be used for immunomodulation, to produce anti-CTLA4 antibodies, to purify CTLA4

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ligands and in screening assays. The CTLA4-Ab fusion proteins are particularly useful when bivalent preparations are preferred, i.e. when crosslinking is desired.

One aspect of the invention pertains to isolated nucleic acid molecules encoding modified CTLA4-immunoglobulin fusion proteins. The nucleic acids of the invention
5 comprise a nucleotide sequence encoding a first peptide having a CTLA4 activity and a nucleotide sequence encoding a second peptide comprising an immunoglobulin constant region which is modified to reduce at least one constant region-mediated biological effector function. A peptide having a CTLA4 activity is defined herein as a peptide
10 having at least one biological activity of the CTLA4 protein, e.g., the ability to bind to the natural ligand(s) of the CTLA4 antigen on immune cells, such as B7-1 and/or B7-2 on B cells, or other known or as yet undefined ligands on immune cells, and inhibit (e.g., block) or interfere with immune cell mediated responses. In one embodiment, the peptide having a CTLA4 activity binds B7-1 and/or B-2 and comprises an extracellular domain of the CTLA4 protein. Preferably, the extracellular domain includes amino acid
15 residues 20-144 of the human CTLA4 protein (amino acid positions 20-144 of SEQ ID NO: 24, 26 and 28).

The present invention also contemplates forms of the extracellular domain of CTLA4 which are expressed without Ig constant regions and are expressed in *E. coli*. These soluble forms of the CTLA4 extracellular domain, although not glycosylated, are
20 fully functional and have similar uses as the CTLA4 immunoglobulin fusion proteins of the invention.

The nucleic acids of the invention further comprise a nucleotide sequence encoding a second peptide comprising an immunoglobulin constant region which is modified to reduce at least one Ig constant region-mediated biological effector function.
25 Preferably, the immunoglobulin constant region comprises a hinge region, a CH2 domain and a CH3 domain derived from C γ 1, C γ 2, C γ 3 or C γ 4. In one embodiment, the constant region segment is altered (e.g., mutated at specific amino acid residues by substitution, deletion or addition of amino acid residues) to reduce at least one IgC region-mediated effector function. In another embodiment, a constant region other than
30 C γ 1 that exhibits reduced IgC region-mediated effector functions relative to C γ 1 is used in the fusion protein. In a preferred embodiment, the CH2 domain is modified to reduce a biological effector function, such as complement activation, Fc receptor interaction, or both complement activation and Fc receptor interaction. For example, to reduce Fc receptor interaction, at least one amino acid residue selected from a hinge link region of
35 the CH2 domain (e.g., amino acid residues at positions 234-237 of an intact heavy chain protein) is modified by substitution, addition or deletion of amino acids. In another

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embodiment, to reduce complement activation ability, a constant region which lacks the ability to activate complement, such as Cy4 or Cy2 is used in the fusion protein (instead of a Cy1 constant region which is capable of activating complement). In another embodiment the variable region of the heavy and light chain is replaced with a polypeptide having CTLA4 activity creating a CTLA4-Ab molecule. In a preferred embodiment the heavy chain constant region of the CTLA4-Ab molecule comprises a hinge region, a CH2 domain and a CH3 domain derived from Cy1, Cy2, Cy3 or Cy4. In a preferred embodiment the light chain constant region of the CTLA4-Ab molecule comprises an Ig signal sequence, the CTLA4 extracellular domain, and the light chain (kappa or lambda) constant domain.

The nucleic acids obtained in accordance with this invention can be inserted into various expression vectors, which in turn direct the synthesis of the corresponding protein in a variety of hosts, particularly eucaryotic cells, such as mammalian or insect cell culture and procaryotic cells, such as *E. coli*. Expression vectors within the scope of the invention comprise a nucleic acid as described herein and a promotor operably linked to the nucleic acid. Such expression vectors can be used to transfect host cells to thereby produce fusion proteins encoded by nucleic acids as described herein.

Another aspect of the invention pertains to isolated CTLA4-immunoglobulin fusion proteins comprising a first peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region which is modified to reduce at least one constant region-mediated biological effector function relative to a CTLA4-IgG1 fusion protein. A preferred CTLA4-immunoglobulin fusion protein comprises an extracellular domain of the CTLA4 protein (e.g., amino acid positions 20-144 of the human CTLA4-immunoglobulin fusion protein shown in SEQ ID NO: 24, 26 and 28) linked to an immunoglobulin constant region comprising a hinge region, a CH2 domain and a CH3 domain derived from Cy1, Cy2, Cy3 or Cy4. A preferred constant domain used to reduce the complement activating ability of the fusion protein is Cy4. In one embodiment, the CH2 domain of the immunoglobulin constant region is modified to reduce at least one biological effector function, such as complement activation or Fc receptor interaction. In a particularly preferred embodiment, the mutated antibody or CTLA4-immunoglobulin fusion protein includes a CH2 domain which is modified by substitution of an amino acid residue at position 234, 235 and/or 237 of an intact heavy chain protein. One example of such a protein is a CTLA4-immunoglobulin fusion protein fused to IgG4 comprising an amino acid sequence shown in SEQ ID NO: 28 or a CTLA4-immunoglobulin fused to IgG1 fusion protein comprising an amino acid sequence shown in SEQ ID NO: 24.

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The mutated antibody or fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject. In certain embodiments the subject proteins can be used to inhibit an interaction between a CTLA4 ligand (e.g., B7-1 and/or B7-2) and a receptor therefor (e.g., CD28 and/or CTLA4) on the surface of a T cell, to thereby suppress cell-mediated immune responses *in vivo*. Inhibition of the CTLA4 ligand/receptor interaction may be useful for both general immunosuppression and to induce antigen-specific T cell tolerance in a subject for use in preventing transplantation rejection (solid organ, skin and bone marrow) or graft versus host disease, particularly in allogeneic bone marrow transplantation. The CTLA4-immunoglobulin fusion proteins can also be used therapeutically in the treatment of autoimmune diseases, allergy and allergic reactions, transplantation rejection and established graft versus host disease in a subject. Moreover, the CTLA4-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-CTLA4 antibodies in a subject, to purify CTLA4 ligands and in screening assays to identify molecules which inhibit the interaction of CTLA4 with a CTLA4 ligand.

Brief Description of the Drawings

Figure 1 is a schematic diagram of the "zip up" polymerase chain reaction (PCR) procedure used to contrast gene fusions.

Figure 2A-B show the amino acid mutations introduced into the hinge and CH2 domains of hCTLA4-IgG1m (panel A) and hCTLA4-IgG4m (panel B). Mutated amino acid residues are underlined.

Figure 3 is a schematic diagram of the expression vector pNRDSH.

Figure 4A-B are graphic representations of competition ELISAs depicting the ability of unlabeled hCTLA4-IgG1 or unlabeled hCTLA4-IgG4m to compete for the binding of biotinylated hCTLA4-IgG1 to hB7-1-Ig (panel A) or hB7-2-Ig (panel B).

Figure 5A-B are graphic representations of Fc receptor binding assays depicting the ability of CTLA4-IgG1 or CTLA4-IgG4 to bind to Fc receptors. In panel A, the ability of unlabeled CTLA4-IgG1 or unlabeled CTLA4-IgG4 to compete for the binding of ¹²⁵I-labeled CTLA4-IgG1 to FcRI-positive U937 cells is depicted. In panel B, the percent specific activity of unlabeled CTLA4-IgG1, CTLA4-IgG4 or hIgG1 used to compete itself for binding to U937 cells is depicted.

Figure 6A-C are graphic representations of complement activation assays depicting the ability of CTLA4-IgG1, CTLA4-IgG4m or anti-B7-1 mAb (4B2) to activate complement-mediated lysis of CHO-B7-1 cells. In panel A, guinea pig complement is used as the complement source. In panel B, human serum is used as the

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complement source. In panel C, control untransfected CHO cells are used as the target for complement-mediated lysis.

Figure 7 is a graphic representation of the binding of CTLA4-IgG1, CTLA4-IgG4m or anti-B7-1 mAb (4B2) to CHO-B7-1 cells, demonstrating that despite the inability of CTLA4-IgG4m to activate complement it can still bind to CHO-B7-1 cells.

Figure 8 is a graphic representation of a competition curve demonstrating that soluble CTLA4 expressed in *E. coli* is functional and competes with unlabeled CTLA4Ig for binding to plate-bound B7-1.

Detailed Description of the Invention

In one embodiment this invention features mutated immunoglobulin molecules which have been modified to reduce more than one immunoglobulin constant (IgC) region-mediated effector function. This invention also features isolated CTLA4-immunoglobulin fusion proteins which have been modified to reduce immunoglobulin constant (IgC) region-mediated effector functions. The invention also features isolated nucleic acids encoding the proteins, methods for producing the mutated antibody and CTLA4-immunoglobulin fusion proteins of the invention and methods for using the mutated antibody and CTLA4-immunoglobulin fusion proteins of the invention for immunomodulation. *E. coli*-expressed forms of CTLA4 are also disclosed. These and other aspects of the invention are described in further detail in the following subsections:

I. Chimeric CTLA4-immunoglobulin Gene Fusions

The invention provides isolated nucleic acids encoding CTLA4-immunoglobulin fusion proteins. The CTLA4-immunoglobulin fusion proteins are comprised of two components: a first peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region which, in certain embodiments is modified to reduce at least one constant region-mediated biological effector function. Accordingly, the isolated nucleic acids of the invention comprise a first nucleotide sequence encoding the first peptide having a CTLA4 activity and a second nucleotide sequence encoding the second peptide comprising an immunoglobulin constant region which, in a preferred embodiment, is modified to reduce at least one constant region-mediated biological effector function. In the case of CTLA4Ig forms, the first and second nucleotide sequences are linked (i.e., in a 5' to 3' orientation by phosphodiester bonds) such that the translational frame of the CTLA4 and IgC coding segments are maintained (i.e., the nucleotide sequences are joined together in-frame). Thus, expression (i.e., transcription

and translation) of the nucleotide sequences produces a functional CTLA4Ig fusion protein. In the case of the CTLA4-Ab fusion proteins, the heavy chain gene is constructed such that the CTLA4 extracellular binding domain is linked to a 5' signal sequence and a 3' immunoglobulin CH1, hinge, CH2, and CH3 domain. CTLA4-light chain constructs are prepared in which an Ig signal sequence, an intron, the CTLA4 extracellular domain, an intron, and the light chain constant domain are linked. The DNA encoding the heavy and light chains is then expressed using an appropriate expression vector as described in the Examples.

The term "nucleic acid" as used herein is intended to include fragments or equivalents thereof. The term "equivalent" is intended to include nucleotide sequences encoding functionally equivalent CTLA4-immunoglobulin fusion proteins, i.e., proteins which have the ability to bind to the natural ligand(s) of the CTLA4 antigen on immune cells, such as B7-1 and/or B7-2 on B cells, and inhibit (e.g., block) or interfere with immune cell mediated responses.

The term "isolated" as used throughout this application refers to a nucleic acid or fusion protein substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An isolated nucleic acid is also free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the organism from which the nucleic acid is derived.

The nucleic acids of the invention can be prepared by standard recombinant DNA techniques. For example, a chimeric CTLA4-immunoglobulin gene fusion can be constructed using separate template DNAs encoding CTLA4 and an immunoglobulin constant region and a "zip up" polymerase chain reaction (PCR) procedure as described in Example 1 and illustrated schematically in Figure 1. Alternatively, a nucleic acid segment encoding CTLA4 can be ligated in-frame to a nucleic acid segment encoding an immunoglobulin constant region using standard techniques. A nucleic acid of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which has been automated in commercially available DNA synthesizers (See e.g., Itakura *et al.* U.S. Patent No. 4,598,049; Caruthers *et al.* U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

The nucleic acid segments of the CTLA4-immunoglobulin gene fusions of the invention are described in further detail below:

A. CTLA4 Gene Segment

An isolated nucleic acid of the invention encodes a first peptide having a CTLA4 activity. The phrase "peptide having a CTLA4 activity" or "peptide having an activity of CTLA4" is used herein to refer to a peptide having at least one biological activity of the CTLA4 protein, i.e., the ability to bind to the natural ligand(s) of the CTLA4 antigen on immune cells, such as B7-1 and/or B7-2 on B cells, or other known or as yet undefined ligands on immune cells, and which, in soluble form, can inhibit (e.g., block) or interfere with immune cell mediated responses. In one embodiment, the CTLA4 protein is a human CTLA4 protein, the nucleotide and amino acid sequences of which are disclosed in Harper, K., et al. (1991) *J. Immunol.* 147:1037-1044 and Dariavich, et al. (1988) *Eur. J. Immunol.* 18(12):1901-1905. In another embodiment, the peptide having a CTLA4 activity binds B7-1 and/or B7-2 and comprises at least a portion of an extracellular domain of the CTLA4 protein. Preferably, the extracellular domain includes amino acid residues 1-125 of the human CTLA4 protein (amino acid positions 20-144 of SEQ ID NO: 24, 26 and 28). CTLA4 proteins from other species (e.g., mouse) are also encompassed by the invention. The nucleotide and amino acid sequences of mouse CTLA4 are disclosed in Brunet, J.F., et al., (1987) *Nature* 328:267-270.

The nucleic acids of the invention can be DNA or RNA. For example, nucleic acid encoding a peptide having a CTLA4 activity may be obtained from mRNA present in activated T lymphocytes. It is also possible to obtain nucleic acid encoding CTLA4 from T cell genomic DNA. For example, the gene encoding CTLA4 can be cloned from either a cDNA or a genomic library in accordance with standard protocols. A cDNA encoding CTLA4 can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNAs can then prepared from the total mRNA. Subsequently, the cDNAs can be inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. Genes encoding CTLA4 can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention (see Example 1). For example, a DNA vector containing a CTLA4 cDNA can be used as a template in PCR reactions using oligonucleotide primers designed to amplify a desired region of the CTLA4 cDNA, e.g., the extracellular domain, to obtain an isolated DNA fragment encompassing this region using standard techniques.

It will be appreciated by those skilled in the art that various modifications and equivalents of the nucleic acids encoding the CTLA4-immunoglobulin fusion proteins of the invention exist. For example, different cell lines can be expected to yield DNA

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- molecules having different sequences of bases. Additionally, variations may exist due to genetic polymorphisms or cell-mediated modifications of the genetic material. Furthermore, the nucleotide sequence of a protein of the invention, such as aCTLA4-immunoglobulin fusion protein, can be modified by genetic techniques to produce
- 5 proteins with altered amino acid sequences that retain the functional properties of CTLA4 (e.g., the ability to bind to B7-1 and/or B7-2). Such sequences are considered within the scope of the invention, wherein the expressed protein is capable of binding a natural ligand of CTLA4 and, when in the appropriate form (e.g., soluble) can inhibit B7:CD28/CTLA4 interactions and modulate immune responses and immune function.
- 10 In addition, it will be appreciated by those of skill in the art that there are other B7-binding ligands and the fusion of these alternative molecules (such as CD28) to form immunoglobulin fusion proteins or expressed in soluble form in *E. coli* is also contemplated by the present invention.

- To express a protein, such as a CTLA4-immunoglobulin fusion protein of the
- 15 invention, the chimeric gene fusion encoding the CTLA4-immunoglobulin fusion protein typically includes a nucleotide sequence encoding a signal sequence which, upon transcription and translation of the chimeric gene, directs secretion of the fusion protein. A native CTLA4 signal sequence (e.g., the human CTLA4 signal sequence disclosed in Harper, K., et al. (1991) *J. Immunol.* 147,1037-1044) can be used or alternatively, a
- 20 heterologous signal sequence can be used. For example, the oncostatin-M signal sequence (Malik N., et al.(1989) *Mol. Cell. Biol.* 9(7), 2847-2853) or an immunoglobulin signal sequence (e.g., amino acid positions 1 to 19 of SEQ ID NO: 24, 26 and 28) can be used to direct secretion of a CTLA4-immunoglobulin fusion protein of the invention. A nucleotide sequence encoding a signal sequence can be incorporated
- 25 into the chimeric gene fusion by standard recombinant DNA techniques, such as by "zip up" PCR (described further in Example 1) or by ligating a nucleic acid fragment encoding the signal sequence in-frame at the 5' end of a nucleic acid fragment encoding CTLA4.

30 *B. Immunoglobulin Gene Segment*

- The CTLA4-immunoglobulin fusion protein of the invention further comprises a second peptide linked to the peptide having a CTLA4 activity. In one embodiment the second peptide comprises a light chain constant region. In a preferred embodiment the light chain is a kappa light chain.

- 35 In another embodiment the second peptide comprises a heavy chain constant region. In a preferred embodiment the constant region comprises an immunoglobulin

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hinge region, a CH2 domain and a CH3 domain. In another embodiment the constant region also comprises a CH1 domain. The constant region is preferably derived from Cy1, Cy2, Cy3 or Cy4. In a preferred embodiment the heavy chain constant region is modified to reduce at least one constant region-mediated biological effector function. In one embodiment, the constant region segment (either Cy1 or another isotype) is altered (e.g., mutated from the wild-type sequence at specific amino acid residues by substitution, deletion or addition of amino acid residues) to reduce at least one IgC region-mediated effector function. The effector functions of this altered fusion protein can be assessed relative to an unaltered IgC region-containing molecule (e.g., a whole antibody or Ig fusion protein). In another embodiment, a constant region other than Cy1 that exhibits reduced IgC region-mediated effector functions is used in the fusion protein. The effector functions of this fusion protein can be assessed relative to a Cy1-containing molecule (e.g., an IgG1 antibody or IgG1 fusion protein). In a particularly preferred embodiment, the fusion protein comprises a constant region other than Cy1 that is also mutated to further reduce effector function. For example, a preferred IgC region is a mutated Cy4 region.

The term "immunoglobulin constant (IgC) region-mediated biological effector function" is intended to include biological responses which require or involve, at least in part, the constant region of an immunoglobulin molecule. Examples of such effector functions include complement activation, Fc receptor interactions, opsonization and phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), release of reactive oxygen intermediates and placental transfer. While such effector functions are desirable in many immune responses, they are undesirable in situations where an immune response is to be downmodulated. The mutated immunoglobulins or fusion proteins of the invention exhibit reduced IgC region-mediated biological effector functions and thus are efficient agents for downregulating immune responses. Additionally, the mutated immunoglobulins or immunoglobulin fusion proteins of the invention display a long plasma half life *in vivo*. The long plasma half-life makes the proteins particularly useful as therapeutic agents.

All immunoglobulins have a common core structure of two identical light and heavy chains held together by disulfide bonds. Both the light chains and the heavy chains contain a series of repeating, homologous units, each about 110 amino acid residues in length, which fold independently in a common globular motif, called an immunoglobulin domain. In each chain, one domain (V) has a variable amino acid sequence depending on the antibody specificity of the molecule. The other domains (C) have a constant sequence common among molecules of the same isotype. Heavy chains

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are designated by the letter of the Greek alphabet corresponding to the overall isotype of the antibody: IgA1 contains $\alpha 1$ heavy chains; IgA2, $\alpha 2$; IgD, δ ; IgE, ϵ ; IgG1, $\gamma 1$; IgG2, $\gamma 2$; IgG3, $\gamma 3$; IgG4, $\gamma 4$; and IgM, μ . Each heavy chain includes four domains; an amino terminal variable, or VH domain which displays the greatest sequence variation among heavy chains and three domains which form the constant region (CH1, CH2 and CH3) in order from the amino to the carboxy terminus of the heavy chain. In γ , α and δ heavy chains, there is a nonglobular region of amino acid sequence, known as the hinge, located between the first and second constant region domains (CH1 and CH2) permitting motion between these two domains.

- 10 To modify a immunoglobulin fusion protein, such as a CTLA4-Ig fusion protein or an antibody such that it exhibits reduced binding to the FcRI receptor, the immunoglobulin constant region segment of the CTLA4-immunoglobulin fusion protein or antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. *et al.* (1991) *J. of Immunol.* 147:2657-2662). Reduction in FcR binding ability of an antibody or immunoglobulin fusion protein will also reduce other effector functions which rely on FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity. To reduce FcR binding, in one embodiment, the constant region is mutated within a region of the CH2 domain referred to as the "hinge link" or "lower hinge" region. This region encompasses amino acid residues 234-239 in a full-length native immunoglobulin heavy chain. It should be appreciated that all IgC region amino acid residue positions described herein refer to the position within the full-length intact native immunoglobulin heavy chain; it will be apparent to those skilled in the art that depending upon the length of the CTLA4 segment used in the CTLA4-immunoglobulin fusion protein, the positions of the corresponding IgC amino acid residues within the fusion protein will vary (Kabat, E.A, T.T. Wu, M. Reid-Miller, H.M. Perry, and K.S. Gottesman eds. (1987) "Sequences of Proteins of Immunological Interest" National Institutes of Health, Bethesda, MD). The hinge link region can be mutated by substitution, addition or deletion of amino acid residues. A preferred mutated antibody or immunoglobulin fusion protein of the invention is one in which the IgG1 constant region has substitution mutations at positions 234, 235 and/or 237 of the C γ 1 segment. Preferably, Leu at 234 is substituted with Ala, Leu at 235 is substituted with Glu and Gly at 237 is substituted with Ala (see Example 1). A preferred mutated antibody or CTLA4-immunoglobulin fusion protein of the invention is one in which IgG4 has substitution mutations at positions 235 and/or 237 of the C γ 4

segment. Preferably, Leu at 235 is substituted with Glu and Gly at 237 is substituted with Ala (see Example 1).

5 In another embodiment, the Fc receptor binding capability of the antibody or immunoglobulin fusion protein is reduced by mutating a region of the CH2 domain referred to as the "hinge-proximal bend" region (amino acid residues at positions 328-333 within a full-length intact heavy chain). This region can be mutated by substitution, addition or deletion of amino acid residues. In a preferred embodiment, position 331 of C γ 1 or C γ 3 is mutated. A preferred mutation in C γ 1 or C γ 3 is substitution of Pro with Ser.

10 To modify an antibody or immunoglobulin fusion protein such that it exhibits reduced complement activation ability, the immunoglobulin constant region segment of the antibody or fusion protein can be mutated at particular regions important for complement activation, such as regions involved in IgC region binding to the C1q component of complement. In one embodiment, one or more residues present within the
15 CH2 domain of IgG subclasses that are involved in C1q binding are altered. In a preferred embodiment, positions 318, 320 and/or 322 are mutated (see Duncan and Winter (1988) *Nature* 332, 738-740). Preferably, Glu at 318 is substituted with Ala or Val, Lys at 320 is substituted with Ala or Gln and/or Lys at 322 is substituted with Ala or Gln.

20 Alternatively, to reduce complement activation by the antibody or immunoglobulin fusion protein, a constant region which lacks the ability to activate complement can be used in the fusion protein. For example, it is known that both IgG1 and IgG3, but not IgG2 and IgG4 activate the classical complement cascade in the presence of human complement. Accordingly, an antibody or immunoglobulin fusion
25 protein utilizing a C γ 4 constant region exhibits reduced complement activation ability relative to a immunoglobulin fusion protein comprising IgG1 (as demonstrated in Example 2).

In yet another embodiment, the hinge region of the IgC segment is altered to inhibit complement activation ability. The hinge regions of the human IgG molecules
30 vary in amino acid sequence and composition as well as length. For example, IgG1, IgG2 and IgG4 have hinge regions consisting of 12 to 15 amino acids, whereas IgG3 has an extended hinge region, consisting of 62 amino acids. The hinge region is believed to be essential for binding with the first component of complement, C1q (see Tan et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:162-166). A number of chimeric human IgG3 and
35 IgG4 molecules with different hinge lengths and amino acid composition have been produced, confirming the role of the hinge region in C1q binding and complement

activation. For example, to reduce or interfere with the ability of an IgG1 or IgG3 antibody or CTLA4-immunoglobulin (IgG1) or CTLA4-immunoglobulin (IgG3) construct to activate complement, it may be necessary to modify, by substitution, addition or deletion, at least one amino acid residue in the hinge region. In one
5 embodiment, the hinge region of Cy1 or Cy3 is substituted with a hinge region derived from Cy2 or Cy4, each of which lack the ability to activate complement.

In certain embodiments the modified antibodies or CTLA4-immunoglobulin fusion proteins of the invention contain multiple modifications, i.e., such that they exhibit reduced Fc receptor binding and reduced complement activation.

10 In addition to modifying the antibodies or immunoglobulin fusion proteins of the invention to reduce IgC region-mediated biological effector functions, the fusion proteins can be further modified for other purposes, e.g., to increase solubility, enhance therapeutic or prophylactic efficacy, or stability (e.g., shelf life *ex vivo* and resistance to proteolytic degradation *in vivo*). Such modified proteins are considered functional
15 equivalents of the antibodies or immunoglobulin fusion proteins as defined herein. For example, amino acid residues of the CTLA4 portion of the fusion protein which are not essential for CTLA4 ligand interaction can be modified by being replaced by another amino acid whose incorporation may enhance, diminish, or not affect reactivity of the fusion protein. Alternatively, a CTLA4-immunoglobulin fusion protein which binds
20 only B7-1 or B7-2 but not both could be created by mutating residues involved in binding to one ligand or the other. Another example of a modification of a CTLA4-immunoglobulin fusion protein is substitution of cysteine residues, preferably with alanine, serine, threonine, leucine or glutamic acid residues, to minimize dimerization via disulfide linkages. A particularly preferred modification is substitution of cysteine
25 residues in the hinge region of the immunoglobulin constant region with serine. In addition, amino acid side chains of an antibody or immunoglobulin fusion protein can be chemically modified.

A particularly preferred embodiment of the invention features a nucleic acid encoding a CTLA4-immunoglobulin fusion protein comprising a nucleotide sequence
30 encoding a first peptide having a CTLA4 activity and a nucleotide sequence encoding a second peptide comprising an IgG4 immunoglobulin constant region, Cy4. Preferably, the nucleic acid is a DNA and the first peptide comprises an extracellular region of CTLA4 which binds B7-1. Such a CTLA4-IgG4 construct can comprise a nucleotide sequence shown in SEQ ID NO: 25 and an amino acid sequence shown in SEQ ID NO:
35 26. In an even more preferred embodiment, the CH2 domain of the Cy4 portion of this CTLA4IgG4 fusion protein is modified to reduce Fc receptor interaction. For example,

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the CH2 domain can be modified by substitution of Leu at position 235 (e.g., with Glu) and/or substitution of Gly at position 237 (e.g., with Ala). A particularly preferred CTLA4-IgG4 fusion protein comprises the extracellular domain of human CTLA4 (i.e., amino acid residues 1-125), has reduced Fc receptor interaction due to two substitutions in the CH2 domain (i.e., substitution of Leu at position 235 with Glu and substitution of Gly at position 237 with Ala). Such a CTLA4-IgG4 fusion protein comprises an amino acid sequence shown in SEQ ID NO: 28 and a nucleotide sequence shown in SEQ ID NO: 27. This construct, referred to as CTLA4-IgG4m, exhibits markedly reduced complement activation ability and FcR binding activity relative to a wild-type CTLA4-IgG1 construct (see Example 2).

Another preferred embodiment of the invention features a nucleic acid encoding a CTLA4-IgG1 fusion protein comprising a nucleotide sequence encoding a first peptide having a CTLA4 activity and a nucleotide sequence encoding a second peptide comprising an immunoglobulin constant region, C_γ1, which is modified to reduce at least one constant region-mediated biological effector functions. Preferably, the nucleic acid is a DNA and the first peptide comprises an extracellular region of CTLA4 which binds B7-1. To reduce Fc receptor interaction the CH2 domain of C_γ1 is modified by substitution of one or more of the following amino acid residues: Leu at position 235; Leu at position 234; and Gly at position 237. A particularly preferred CTLA4-IgG1 fusion protein comprises the extracellular domain of human CTLA4 (i.e., amino acid residues 1-125), has reduced Fc receptor interaction due to three substitutions in the CH2 domain (i.e., substitution of Leu at position 234 with Ala, substitution of Leu at position 235 with Glu and substitution of Gly at position 237 with Ala). Such a CTLA4-IgG1 fusion protein, referred to herein as CTLA4-IgG1m, comprises an amino acid sequence shown in SEQ ID NO: 24 and a nucleotide sequence shown in SEQ ID NO: 23.

Nucleic acid encoding a peptide comprising an immunoglobulin constant region can be obtained from human immunoglobulin mRNA present in B lymphocytes. It is also possible to obtain nucleic acid encoding an immunoglobulin constant region from B cell genomic DNA. For example, DNA encoding C_γ1 or C_γ4 can be cloned from either a cDNA or a genomic library or by polymerase chain reaction (PCR) amplification in accordance with protocols herein described. The nucleic acids of the invention can be DNA or RNA. A preferred nucleic acid encoding an immunoglobulin constant region comprises all or a portion of the following: the DNA encoding human C_γ1 (Takahashi, N.S. et al. (1982) *Cell* 29:671-679), the DNA encoding human C_γ2 (Kabat, E.A., T.T. Wu, M. Reid-Miller, H.M. Perry, and K.S. Gottesman eds. (1987) "Sequences of

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Proteins of Immunological Interest" National Institutes of Health, Bethesda, MD); the DNA encoding human C γ 3 (Huck, S., et al. (1986) *Nucl. Acid Res.* 14:1779); and the DNA encoding human C γ 4 (Kabat et al., *supra*).

5 A number of processes are known in the art for modifying a nucleotide or amino acid sequence to thereby mutate the IgC regions as described herein. For example, mutations can be introduced into a DNA by any one of a number of methods, including those for producing simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases, to generate mutated antibodies or CTLA4-immunoglobulin fusion proteins of the invention and equivalents
10 thereof. Preferably, amino acid substitutions, deletions or additions, such as in the CH2 domain of the immunoglobulin constant region, are created by PCR mutagenesis as described in Example 1 or by standard site-directed mutagenesis. Site directed mutagenesis systems are well known in the art. For example, protocols and reagents can be obtained commercially from Amersham International PLC, Amersham, U.K.

15

II. Expression Vectors and Host Cells

The mutated antibodies or immunoglobulin fusion proteins of the invention can be expressed by incorporating a gene, such as a chimeric CTLA4-immunoglobulin fusion gene described herein, into an expression vector and introducing the expression
20 vector into an appropriate host cell. Accordingly, the invention further pertains to expression vectors containing a nucleic acid encoding a mutated antibody gene, or portion thereof or a immunoglobulin fusion protein and to host cells into which such expression vectors have been introduced. An expression vector of the invention, as described herein, typically includes nucleotide sequences encoding the mutated antibody
25 gene or immunoglobulin fusion protein operably linked to at least one regulatory sequence. "Operably linked" is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence in a host cell (or by a cell extract). Regulatory sequences are art-recognized and can be selected to direct expression of the desired protein in an appropriate host cell.
30 The term regulatory sequence is intended to include promoters, enhancers, polyadenylation signals and other expression control elements. Such regulatory sequences are known to those skilled in the art and are described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It should be understood that the design of the expression vector may depend on
35 such factors as the choice of the host cell to be transfected and/or the type and/or amount of protein desired to be expressed.

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An expression vector of the invention can be used to transfect cells, either procaryotic or eucaryotic (e.g., mammalian, insect or yeast cells) to thereby produce fusion proteins encoded by nucleotide sequences of the vector. Expression in procaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters. Certain *E. coli* expression vectors (so called fusion-vectors) are designed to add a number of amino acid residues to the expressed recombinant protein, usually to the amino terminus of the expressed protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the target recombinant protein; and 3) to aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. Examples of fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia) and pMAL (New England Biolabs, Beverly, MA) which fuse glutathione S-transferase and maltose E binding protein, respectively, to the target recombinant protein. Accordingly, the subject gene or fusion gene, e.g., a chimeric CTLA4-immunoglobulin fusion gene, may be linked to additional coding sequences in a procaryotic fusion vector to aid in the expression, solubility or purification of the fusion protein. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the target recombinant protein to enable separation of the target recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from the T7 gn10-lac 0 fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize expression of the subject recombinant protein in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy would be to alter the nucleotide sequence of the antibody immunoglobulin fusion protein to be inserted into an expression vector so that the individual codons for each amino acid would be those preferentially utilized in

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highly expressed *E. coli* proteins (Wada et al., (1992) *Nuc. Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences are encompassed by the invention and can be carried out by standard DNA synthesis techniques.

5 In another preferred embodiment a soluble CTLA4 extracellular domain is expressed in *E. coli* using an appropriate expression vector. These forms, although not glycosylated, remain fully functional and represent an advantage because of the ease with which bacterial cells are grown.

Alternatively, a mutated antibody or immunoglobulin fusion protein can be expressed in a eucaryotic host cell, such as mammalian cells (e.g., Chinese hamster
10 ovary cells (CHO) or NS0 cells), insect cells (e.g., using a baculovirus vector) or yeast cells. Other suitable host cells may be found in Goeddel, (1990) *supra* or are known to those skilled in the art. Eucaryotic, rather than procaryotic, expression of the subject protein may be preferable since expression of eucaryotic proteins in eucaryotic cells can lead to partial or complete glycosylation and/or formation of relevant inter- or intra-
15 chain disulfide bonds of a recombinant protein. For expression in mammalian cells, the expression vector's control functions are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. To express a CTLA4-immunoglobulin fusion protein in mammalian cells, generally COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used
20 in conjunction with such vectors as pCDM8 (Seed, B., (1987) *Nature* 329:840) for transient amplification/expression, while CHO (dhfr⁻ Chinese Hamster Ovary) cells are used with vectors such as pMT2PC (Kaufman et al. (1987), *EMBO J.* 6:187-195) for stable amplification/expression in mammalian cells. A preferred cell line for production of recombinant protein is the NS0 myeloma cell line available from the ECACC
25 (catalog #85110503) and described in Galfre, G. and Milstein, C. ((1981) *Methods in Enzymology* 73(13):3-46; and *Preparation of Monoclonal Antibodies: Strategies and Procedures*, Academic Press, N.Y., N.Y). Examples of vectors suitable for expression of recombinant proteins in yeast (e.g., *S. cerevisiae*) include pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943),
30 pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39).

35 Vector DNA can be introduced into procaryotic or eucaryotic cells via conventional transformation or transfection techniques such as calcium phosphate or

calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

5 For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate DNA into their genomes. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable
10 markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduced into a host cell on the same plasmid as the gene of interest or may be introduced on a separate plasmid. Cells containing the gene of interest can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells
15 die). The surviving cells can then be screened for production of the antibody of interest or CTLA4-immunoglobulin fusion proteins by, for example, immunoprecipitation from cell supernatant with an anti-CTLA4 monoclonal antibody.

The invention also features methods of producing mutated antibodies or immunoglobulin fusion proteins. For example, a host cell transfected with a nucleic
20 acid vector directing expression of a nucleotide sequence encoding a CTLA4-immunoglobulin fusion protein can be cultured in a medium under appropriate conditions to allow expression of the protein to occur. In one embodiment, a recombinant expression vector containing DNA encoding a CTLA4-IgG1 fusion protein having modified constant region-mediated effector functions is produced. In another
25 embodiment, a recombinant expression vector containing DNA encoding a CTLA4-IgG4 fusion protein having modified constant region-mediated effector functions is produced. In addition, one or more expression vectors containing DNA encoding, for example, a CTLA4-IgG1 fusion protein and another fusion protein such as a CTLA4-IgG4 fusion protein can be used to transfect a host cell to coexpress these fusion
30 proteins. Fusion proteins produced by recombinant technique may be secreted and isolated from a mixture of cells and medium containing the protein. Alternatively, the protein may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable mediums for cell culture are well known in the art. Protein can be isolated
35 from cell culture medium, host cells, or both using techniques known in the art for purifying proteins.

III. Isolation and Characterization of Mutated antibodies or Immunoglobulin Fusion Proteins

Another aspect of the invention pertains to mutated antibodies or fusion proteins having modified effector functions compared to the non-mutated form, for example, CTLA4-immunoglobulin fusion proteins having modified effector functions compared to a CTLA4-IgG1 protein. Such proteins comprise a first peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region which is modified to reduce at least one constant region-mediated biological effector function relative to a CTLA4-IgG1 fusion protein. A peptide having a CTLA4 activity has been previously defined herein. In a preferred embodiment, the first peptide comprises an extracellular domain of the human CTLA4 protein (e.g., amino acid residues 20-144 of SEQ ID NO: 24, 26 and 28) and binds B7-1 and/or B7-2. The second peptide comprising an immunoglobulin constant region preferably includes a hinge region, a CH2 domain and a CH3 domain derived from Cy1, Cy2, Cy3, or Cy4. Typically, the CH2 domain is modified to reduce constant region-mediated biological effector functions, such as complement activation and/or Fc receptor interaction as previously described in detail herein.

Another embodiment of the invention provides a substantially pure preparation of a mutated antibody or immunoglobulin fusion protein as described herein. Such a preparation is substantially free of proteins and peptides with which the protein naturally occurs in a cell or with which it naturally occurs when secreted by a cell.

Such antibodies or immunoglobulin fusion proteins, expressed in mammalian cells or elsewhere, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fractionation column chromatography (e.g., ion exchange, gel filtration, electrophoresis, affinity chromatography, etc.) and ultimately, crystallization (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology*, 22:233-577 (1971)). Preferably, the antibodies or immunoglobulin fusion proteins are purified using an immobilized protein A column (Repligen Corporation, Cambridge, MA). Once purified, partially or to homogeneity, the recombinantly produced antibody or immunoglobulin fusion proteins or portions thereof can be utilized in compositions suitable for pharmaceutical administration as described in detail herein.

In one embodiment the CTLA4-immunoglobulin fusion protein is an antibody form in which the heavy and light chains have been replaced with the extracellular domain of CTLA4. This molecule has a different valency and higher affinity for

CTLA4 ligands, thus making it possible to obtain similar results while using less of the agent. The fact that this molecule has a true antibody tail which is fully glycosylated means that numerous cell lines for producing the CTLA4Ab fusion protein and data regarding the clinical use of antibodies may be relied on.

- 5 Screening of antibodies or immunoglobulin fusion proteins which have been modified to reduce at least one constant region-mediated biological effector function as described herein can be accomplished using one or more of several different assays which measure different effector functions. For example, to identify a CTLA4-immunoglobulin fusion protein having reduced Fc receptor interaction, a monomeric
- 10 IgG binding assay can be conducted (see Example 2). A cell which expresses an Fc receptor, such as a mononuclear phagocyte or the U937 cell line (FcγRI expression), a hematopoietic cell (FcγRII expression; Rosenfeld, S.I., et al. (1985) *J. Clin. Invest.* 76:2317-2322) or a neutrophil (FcγRIII expression; Fleit, H.B., et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:3275-3279 and Petroni, K.C., et al. (1988) *J. Immunol.* 140:3467-
- 15 3472) is contacted with, for example, ¹²⁵I-labeled immunoglobulin of the IgG1 isotype in the presence or absence of a modified CTLA4-immunoglobulin fusion protein of the invention and in the presence or absence of an appropriate control molecule (e.g., an unlabeled IgG1 antibody or a CTLA4-IgG1 fusion protein). The amount of ¹²⁵I-labeled IgG1 bound to the cells and/or the amount of free ¹²⁵I-labeled IgG in the supernatant is
- 20 determined. A CTLA4-immunoglobulin fusion protein having reduced Fc receptor binding is identified by a reduced ability (or lack of ability) to inhibit binding of the ¹²⁵I-labeled IgG1 to the cells (relative to the control molecule). Monomeric IgG binding assay are described further in Lund, J., et al. (1991) *J. Immunol.* 147:2657-2662; and Woof, J.M., et al. (1986) *Mol. Immunol.* 23:319.
- 25 To identify a CTLA4-immunoglobulin fusion protein with a reduced ability to activate the complement cascade, a complement activation assay such as that described in Example 2 can be used. In this assay, a cell which expresses a CTLA4 ligand (e.g., B7-1 or B7-2) on its surface is loaded with a detectable substance, e.g., a fluorescent dye, and then contacted with the CTLA4-immunoglobulin fusion protein and a
- 30 complement source (e.g., purified guinea pig complement or human serum as a source of human complement). Cell lysis, as determined by release of the fluorescent dye from the cells, is determined as an indication of activation of the complement cascade upon binding of CTLA4-immunoglobulin to the CTLA4 ligand on the cell surface. Cells which do not express a CTLA4 ligand on their surface are used as a negative control. A
- 35 CTLA4-immunoglobulin fusion protein with reduced ability (or lack of the ability) to activate complement relative to an appropriate control molecule (e.g., anti-B7-1

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antibody of the IgG1 isotype or a CTLA4-IgG1 fusion protein) is identified by a reduction in or absence of cell lysis of labeled, CTLA4 ligand positive cells when incubated in the presence of the CTLA4-immunoglobulin fusion protein of the invention and complement compared to cells incubated in the presence of the control molecule and complement.

In another complement activation assay, the ability of a CTLA4-immunoglobulin fusion protein to bind the first component of the complement cascade, C1q, is assessed. For example, C1q binding can be determined using a solid phase assay in which ¹²⁵I-labeled human C1q is added to an amount of CTLA4-immunoglobulin fusion protein complexed with a CTLA4 ligand, such as B7-1 or B7-2, and the amount of bound ¹²⁵I-labeled human C1q quantitated. A CTLA4-immunoglobulin fusion protein having a reduced complement activation activity (or lack of complement activation activity) is identified by a reduction in or absence of the ability to bind the ¹²⁵I-labeled human C1q relative to an appropriate control molecule (e.g., an IgG1 antibody or a CTLA4-IgG1 fusion protein). C1q binding assays are described further in Tan, L.K., et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:162-166; and Duncan, A.R. and G. Winter (1988) *Nature* 332:738-740.

Additional assays for other immunoglobulin constant region-mediated effector functions, such as opsonization and phagocytosis, antibody-dependent cellular cytotoxicity and release of reactive oxygen intermediates, have been described in the art and are known to the skilled artisan.

Screening for CTLA4-immunoglobulin fusion proteins which have a CTLA4 activity as described herein can be accomplished using one or more of several different assays. For example, the fusion proteins can be screened for specific reactivity with an anti-CTLA4 antibody (e.g., a monoclonal or polyclonal anti-CTLA4 antibody) or with a soluble form of a CTLA4 ligand, such as a B7-1 or B7-2 fusion protein (e.g., B7-1Ig or B7-2Ig). For example, appropriate cells, such as CHO or NS0 cells, can be transfected with a DNA encoding a CTLA4-immunoglobulin fusion protein and the cell supernatant analyzed for expression of the resulting fusion protein using an anti-CTLA4 monoclonal antibody or B7-1Ig or B7-2Ig fusion protein in a standard immunoprecipitation assay. Alternatively, the binding of a CTLA4-immunoglobulin fusion protein to a cell which expresses a CTLA4 ligand, such as a B7-1 or B7-2, on its surface can be assessed. For example, a cell expressing a CTLA4 ligand, such as a CHO cell transfected to express B7-1, is contacted with the CTLA4-immunoglobulin fusion protein and binding detected by indirect immunostaining using, for example, a FITC-conjugated reagent (e.g., goat anti-mouse Ig serum for murine monoclonal antibodies or goat anti-human IgG serum

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for fusion proteins) and fluorescence analyzed by FACS® analysis(Becton Dickinson & Co., Mountain View, CA).

Other suitable assays take advantage of the functional characteristics of the CTLA4-immunoglobulin fusion protein. As previously set forth, the ability of T cells to synthesize cytokines depends not only on occupancy or cross-linking of the T cell receptor for antigen ("the primary activation signal provided by, for example antigen bound to an MHC molecule, anti-CD3, or phorbol ester to produce an "activated T cell"), but also on the induction of a costimulatory signal, in this case, by interaction of a B7 family protein (e.g., B7-1 or B7-2) with its ligand (CD28 and/or CTLA4) on the surface of T cells. The B7:CD28/CTLA4 interaction has the effect of transmitting a signal to the T cell that induces the production of increased levels of cytokines, particularly of interleukin-2, which in turn stimulates the proliferation of the T lymphocytes. In one embodiment, the CTLA4-immunoglobulin fusion proteins of the invention have the functional property of being able to inhibit the B7:CD28/CTLA4 interaction. Accordingly, other screening assays for identifying a functional CTLA4-immunoglobulin fusion protein involve assaying for the ability of the fusion protein to inhibit synthesis of cytokines, such as interleukin-2, interleukin-4 or other known or unknown novel cytokines and/or the ability to inhibit T cell proliferation by T cells which have received a primary activation signal.

The ability of a CTLA4-immunoglobulin fusion protein of the invention to inhibit or block an interaction between a B7 family protein (e.g., B7-1 or B7-2) with its receptor on T cells (e.g., CD28 and/or CTLA4) can be assessed in an *in vitro* T cell culture system by stimulating T cells with a source of ligand (e.g., cells expressing B7-1 and/or B7-2 or a secreted form of B7-1 and/or B7-2) and a primary activation signal such as antigen in association with Class II MHC (or alternatively, anti-CD3 antibodies or phorbol ester) in the presence or absence of the CTLA4-immunoglobulin fusion protein. The culture supernatant is then assayed for cytokine production, such as interleukin-2, gamma interferon, or other known or unknown cytokine. For example, any one of several conventional assays for interleukin-2 can be employed, such as the assay described in *Proc. Natl. Acad. Sci. USA*, 86:1333 (1989). An assay kit for interferon production is also available from Genzyme Corporation (Cambridge, MA.). T cell proliferation can be measured *in vitro* by determining the amount of ³H-labeled thymidine incorporated into the replicating DNA of cultured cells. The rate and amount of DNA synthesis and, in turn, the rate of cell division can thus be quantified. A lack of or reduction in the amount of cytokine production and/or T cell proliferation by stimulated T cells upon culture with a CTLA4-immunoglobulin fusion protein of the

invention indicates that the fusion protein is capable of inhibiting the delivery of a costimulatory signal to the T cell by inhibiting an interaction between a CTLA4 ligand (e.g., B7-1 and/or B7-2) and a receptor therefor (e.g., CD28 and/or CTLA4).

The ability of the CTLA4Ig fusion protein to induce antigen-specific T cell unresponsiveness or anergy can also be assessed using the *in vitro* T cell culture system described above. Following stimulation of the T cells with a specific antigen bound to MHC molecules on an antigen presenting cell surface and CTLA4 ligand (e.g., B7-1 on the antigen presenting cell surface) in the presence of CTLA4-immunoglobulin fusion protein, the T cells are subsequently restimulated with the antigen in the absence of CTLA4-immunoglobulin fusion protein. A lack of cytokine production and/or T cell proliferation upon antigenic restimulation by T cells previously treated with a CTLA4-immunoglobulin fusion protein of the invention indicates that the fusion protein has induced a state of antigen-specific anergy or non-responsiveness in the T cells. See, e.g., Gimmi, C.D. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6586-6590; and Schwartz (1990) *Science* 248:1349-1356, for assay systems that can be used to examine T cell unresponsiveness in accordance with the present invention.

In yet another assay, the ability of a CTLA4-immunoglobulin fusion protein of the invention to inhibit T cell dependent immune responses *in vitro* is determined. The effect of a CTLA4-immunoglobulin fusion protein on T_H -induced immunoglobulin production by B cells can be assessed by contacting antigen-specific $CD4^+$ T cells with syngeneic antigen-specific B cells, antigen and the CTLA4-immunoglobulin fusion protein. The cell culture supernatant is assayed for the production of immunoglobulin, such as IgG or IgM, using, for example, a solid phase ELISA or a standard plaque assay. Inhibition of B cell immunoglobulin production by treatment of the culture with the CTLA4-immunoglobulin fusion protein indicates that the protein is capable of inhibiting T helper cell responses and, consequently, T cell dependent B cell responses.

IV. Compositions of Antibodies or Immunoglobulin Fusion Proteins

The mutated antibodies or CTLA4-immunoglobulin fusion proteins of the invention can be incorporated into compositions suitable for administration to subjects to thereby modulate immune responses or for other purposes (e.g., antibody production). The mutated antibody or CTLA4-immunoglobulin fusion protein in such compositions is in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include living

organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, monkeys, dogs, cats, mice, rats, and transgenic species thereof. Administration of a CTLA4-immunoglobulin fusion protein as described herein can be in any pharmacological form including a therapeutically active amount of protein and a pharmaceutically acceptable carrier. Administration of a therapeutically active amount of the therapeutic compositions of the invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a CTLA4-immunoglobulin fusion protein may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of protein to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound (e.g., CTLA4-immunoglobulin fusion protein) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

To administer an antibody or immunoglobulin fusion protein by other than parenteral administration, it may be necessary to coat the protein with, or co-administer the protein with, a material to prevent its inactivation. For example, a CTLA4-immunoglobulin fusion protein may be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound, such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

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Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists.

5 It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example,

10 by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars,

15 polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., CTLA4-immunoglobulin fusion protein) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In

20 the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., peptide) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the protein

30 may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any

35 conventional media or agent is incompatible with the active compound, use thereof in

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the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used
5 herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active
10 compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

V. Uses of CTLA4-immunoglobulin Fusion Proteins Having Reduced IgC Region-Mediated Biological Effector Functions

A. Immunomodulation

Given the role of CTLA4 ligands, such as B7-1 and B7-2, in T cell costimulation and the structure and function of the CTLA4-immunoglobulin fusion proteins disclosed
20 herein, the invention provides methods for downregulating immune responses. The reduced IgC-region mediated biological effector functions exhibited by the mutated CTLA4-immunoglobulin fusion proteins of the invention compared to a CTLA4-IgG1 fusion protein may result in more effective downregulation of immune responses *in vivo* without unwanted side effects (e.g., complement activation, antibody-dependent cellular
25 cytotoxicity, etc.) than if a CTLA4-IgG1 fusion protein were used. For example, improvements in mutated forms of CTLA4-immunoglobulin fusion proteins can be assessed by a variety of assays known to those skilled in the art, including various animal organ (heart, liver, kidney, bone marrow) transplantation models and in animal autoimmune disease models including, but not limited to lupus, multiple sclerosis,
30 diabetes, and arthritis models.

Downregulation of an immune response by a CTLA4-immunoglobulin fusion protein of the invention may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The functions of activated T cells, such as T cell proliferation and cytokine
35 (e.g., IL-2) secretion, may be inhibited by suppressing T cell responses or by inducing specific tolerance in T cells, or both. Immunosuppression of T cell responses is

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generally an active process which requires continuous exposure of the T cells to the suppressive agent and is often not antigen-specific. Tolerance, which involves inducing non-responsiveness or anergy in T cells, is distinguishable from immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, T cell unresponsiveness or anergy can be demonstrated by the lack of a T cell response upon reexposure to specific antigen in the absence of the tolerizing agent. Immunosuppression and/or T cell unresponsiveness is achieved by blocking the interaction of a CTLA4 ligand on an antigen presenting cell with CTLA4 itself and/or with another receptor for the CTLA4 ligand (e.g., CD28) on the surface of a T cell, e.g., blocking the interaction of a B7 family protein, such as B7-1 and/or B7-2, with a counter-receptor, such as CD28 or CTLA4, on the surface of a T cell. The term "antigen presenting cell" is intended to include B lymphocytes, professional antigen presenting cells (e.g., monocytes, dendritic cells, Langerhan cells) and others cells (e.g., keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes) which can present antigen to T cells. The CTLA4-immunoglobulin fusion proteins of the invention can be used to inhibit CTLA4 ligand/receptor interactions in many clinical situations, as described further below.

1. Organ Transplantation/GVHD: Inhibition of T cell responses by a CTLA4-immunoglobulin fusion protein of the invention is useful in situations of cellular, tissue, skin and organ transplantation and in bone marrow transplantation (e.g., to inhibit graft-versus-host disease (GVHD)). For example, inhibition of T cell proliferation and/or cytokine secretion may result in reduced tissue destruction in tissue transplantation and induction of antigen-specific T cell unresponsiveness may result in long-term graft acceptance without the need for generalized immunosuppression. Typically, in tissue transplants, rejection of the graft is initiated through its recognition as foreign by T cells, followed by an immune reaction that destroys the graft. Administration of a CTLA4-immunoglobulin fusion protein of the invention to a transplant recipient inhibits triggering of a costimulatory signal in alloantigen-specific T cells, thereby inhibiting T cell responses to alloantigens and, moreover, may induce graft-specific T cell unresponsiveness in the recipient. The transplant recipient can be treated with the CTLA4-immunoglobulin fusion protein alone or together with one or more additional agents that inhibit the generation of stimulatory signals in the T cells (e.g., anti-B7-1 and/or anti-B7-2 antibodies, an anti-IL-2 receptor antibody) or induce general immunosuppression (e.g., cyclosporin A or FK506).

Use of a CTLA4-immunoglobulin fusion protein to inhibit triggering of a costimulatory signal in T cells can similarly be applied to the situation of bone marrow

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transplantation to specifically inhibit the responses of alloreactive T cells present in donor bone marrow and thus inhibit GVHD. A CTLA4-immunoglobulin fusion protein can be administered to a bone marrow transplant recipient to inhibit the alloreactivity of donor T cells. Additionally or alternatively, donor T cells within the bone marrow graft can be tolerized to recipient alloantigens *ex vivo* prior to transplantation. For example, donor bone marrow can be cultured with cells from the recipient (e.g., irradiated hematopoietic cells) in the presence of a CTLA4-immunoglobulin fusion protein of the invention prior to transplantation. Additional agents that inhibit the generation of stimulatory signals in the T cells (e.g., anti-B7-1 and/or anti-B7-2 antibodies, an anti-IL-2R antibody etc., as described above) can be included in the culture. After transplantation, the recipient may be further treated by *in vivo* administration of CTLA4-immunoglobulin (alone or together with another agent(s) which inhibits the generation of a costimulatory signal in T cells in the recipient or inhibits the production or function of a T cell growth factor(s) (e.g., IL-2) in the recipient).

The efficacy of a particular CTLA4-immunoglobulin fusion protein in inhibiting organ transplant rejection or GVHD can be assessed using animal models that may be predictive of efficacy in humans. Given the homology between CTLA4 molecules of different species, the functionally important aspects of CTLA4 are believed to be conserved structurally among species thus allowing animal systems to be used as models for efficacy in humans. Examples of appropriate systems which can be used include allogeneic cardiac grafts in rats and xenogeneic pancreatic islet cell grafts in mice, both of which have been used to examine the immunosuppressive effects of CTLA4-IgG1 fusion proteins *in vivo* as described in Lenschow et al., *Science*, 257: 789-792 (1992) and Turka et al., *Proc. Natl. Acad. Sci. USA*, 89: 11102-11105 (1992). In addition, murine models of GVHD (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 846-847) can be used to determine the effect of treatment with a CTLA4-immunoglobulin fusion protein of the invention on the development of that disease.

As an illustrative embodiment, a CTLA4-immunoglobulin fusion protein of the invention can be used in a rat model of organ transplantation to ascertain the ability of the fusion protein to inhibit alloantigen responses *in vivo*. Recipient Lewis rats receive a Brown-Norway rat strain cardiac allograft which is anastomosed to vessels in the neck as described in Bolling, S.F. et al., *Transplant.* 453:283-286 (1992). Grafts are monitored for mechanical function by palpation and for electrophysiologic function by electrocardiogram. Graft rejection is said to occur on the last day of palpable contractile function. As an initial test, animals are treated with daily injections of a CTLA4-

immunoglobulin fusion protein of interest, an isotype-matched control Ig fusion protein and/or CTLA4-IgG1 (for comparison purposes) for 7 days. Fusion proteins are administered at a dosage range between approximately 0.015 mg/day and 0.5 mg/day. Untreated Lewis rats typically reject heterotopic Brown-Norway allografts in about 7 days. The rejection of allografts by fusion protein-treated animals is assessed in comparison to untreated controls.

An untreated animal and a fusion protein-treated animal are sacrificed for histological examination. Cardiac allografts are removed from the untreated animal and the treated animal four days after transplantation. Allografts are fixed in formalin, and tissue sections are stained with hematoxylin-eosin. The heart tissue of the untreated and treated animals is examined histologically for severe acute cellular rejection including a prominent interstitial mononuclear cell infiltrate with edema formation, myocyte destruction, and infiltration of arteriolar walls. The effectiveness of the fusion protein treatment in inhibiting graft rejection is supported by a lack of an acute cellular rejection in the heart tissue of the fusion protein treated animals.

To determine whether fusion protein therapy establishes long term graft acceptance that persists following treatment, animals treated for 7 days with daily injections of fusion protein are observed without additional therapy until cessation of graft function. Graft survival is assessed daily as described above. Allografts are examined histologically from animals in which the graft stops functioning as described above. Induction of graft tolerance by fusion protein treatment is indicated by the continued functioning of the graft following the cessation of treatment with the fusion protein.

After prolonged graft acceptance, a fusion protein-treated animal can be sacrificed and the lymphocytes from the recipient can be tested for their functional responses. These responses are compared with those of lymphocytes from a control (non-transplanted) Lewis rat, and results are normalized as a percentage of the control response. The T cell proliferative response to ConA and to cells from a Brown-Norway rat and a third party ACI rat can be examined. Additionally, the thymus and spleen from the untreated and treated animals can be compared in size, cell number and cell type (e.g. by flow cytometric analyses of thymus, lymph nodes and spleen cells). Specific nonresponsiveness in the treated animals to alloantigens, as a result of specific clonal deletion of alloreactive cells, is indicated by the ability of the T cells to respond to ConA and third party stimulators (e.g., ACI rat cells) but not to Brown-Norway rat cells. Prolonged acceptance of allografts, including continued graft acceptance following CTLA4-immunoglobulin treatment, in this model system may be predictive of the

therapeutic efficacy of the CTLA4-immunoglobulin fusion proteins of the invention in human transplant situations.

2. Autoimmune Diseases: Inhibition of T cell responses by a CTLA4-immunoglobulin fusion protein of the invention may also be therapeutically useful for treating autoimmune diseases. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue (i.e., reactive against autoantigens) and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive T cells thus may reduce or eliminate disease symptoms. Administration of a CTLA4-immunoglobulin fusion protein of the invention to a subject suffering from or susceptible to an autoimmune disorder may inhibit autoantigen-specific T cell responses and induce autoantigen-specific T cell unresponsiveness, thereby inhibiting or preventing production of autoantibodies or T cell-derived cytokines which may be involved in the disease process.

To treat an autoimmune disorder, a CTLA4-immunoglobulin fusion protein of the invention is administered to a subject in need of treatment. For autoimmune disorders with a known autoantigen, it may be desirable to coadminister the autoantigen with the CTLA4-immunoglobulin to the subject. This method can be used to treat a variety of autoimmune diseases and disorders having an autoimmune component, including diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

The efficacy of a CTLA4-immunoglobulin fusion protein of the invention in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythematosus in

5 MRL/lpr/lpr mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856).

Experimental Autoimmune Encephalomyelitis (EAE) is a rodent and primate model for multiple sclerosis. In an illustrative embodiment utilizing the passive EAE

10 model, donor mice are immunized with 0.4 mg Myelin Basic Protein (MBP) in Complete Freund's Adjuvant (CFA), divided over four quadrants. The draining axillary and inguinal lymph nodes are removed eleven days later. Lymph node cells (4×10^6 /ml) are plated in 2 ml cultures in 24 well plates, in the presence of 25 μ g/ml MBP. After four days in culture, 30×10^6 of the treated cells are injected into the tail vein of

15 each naive, syngeneic recipient mouse.

The recipient mice develop a remitting, relapsing disease and are evaluated utilizing the following criteria:

- | | | |
|----|---|---|
| | 0 | normal, healthy |
| | 1 | limp tail, incontinence; occasionally the first sign of the disease is a "tilt" |
| 20 | 2 | hind limb weakness, clumsiness |
| | 3 | mild paraparesis |
| | 4 | severe paraparesis |
| | 5 | quadriplegia |
| | 6 | death |

25 Using the passive model of EAE, the effect of CTLA4-immunoglobulin treatment of the donor cells on resulting disease severity in a recipient animal is tested in mice (e.g., the PLSJLF1/J strain). Culture of lymph node cells *in vitro* with MBP is performed either in the presence or the absence of about 30 μ g/ml of a CTLA4-immunoglobulin fusion protein of the invention, an isotype matched control Ig fusion

30 protein or CTLA4IgG1 (for comparison purposes). The treated cells are then introduced into a syngeneic recipient mouse. The effect of fusion protein treatment of donor cells on the severity of the recipient's first episode of disease as compared to mice receiving untreated cells can be determined using the above-described criteria to assess disease severity. In addition, ensuing relapses in the mice receiving fusion protein-treated cells

35 versus untreated cells can be assessed using the above-described criteria.

The effect of treating both the donor mice and the cultured donor cells with CTLA4-immunoglobulin on the clinical disease severity in the recipient can further be assessed. In these experiments, donor mice (e.g., of the SJL/J strain) immunized with MBP are given either 100 µg of CTLA4-immunoglobulin fusion protein, an isotype matched control Ig fusion protein or CTLA4-IgG1 (for comparison) intraperitoneally each day for eleven days. Cells are then isolated from lymph nodes of these donors and cultured with MBP *in vitro* in the presence of either 30 µg/ml of CTLA4-immunoglobulin fusion protein or control fusion proteins. The treated cells are then introduced into a syngeneic recipient. The effect of fusion protein treatment on the severity of the ensuing disease in the recipient is then assessed using the above-described criteria.

Studies using a direct (active) model of EAE can also be conducted. In these experiments, a CTLA4-immunoglobulin fusion protein of the invention or control fusion protein is directly administered to mice immunized with MBP and treated with pertussis toxin (PT). Mice (e.g., the PLSJLFI/J strain) are immunized with MBP on day 0, injected with PT intravenously on days 0 and 2, and given either a CTLA4-immunoglobulin fusion protein of the invention or a control fusion protein on days 0 to 7. The effect of direct fusion protein treatment of the MBP-immunized mice on the severity of the ensuing disease is then assessed using the above-described criteria. A reduced severity in disease symptoms in the passive and/or active EAE model as a result of CTLA4-immunoglobulin treatment may be predictive of the therapeutic efficacy of the CTLA4-immunoglobulin fusion proteins of the invention in human autoimmune diseases.

3. Allergy:

The IgE antibody response in atopic allergy is highly T cell dependent and, thus, inhibition of CTLA4 ligand/receptor induced T cell activation may be useful therapeutically in the treatment of allergy and allergic reactions. A CTLA4-immunoglobulin fusion protein of the invention can be administered to an allergic subject to inhibit T cell mediated allergic responses in the subject. Inhibition of costimulation of T cells through inhibition of a CTLA4 ligand/receptor interaction may be accompanied by exposure to allergen in conjunction with appropriate MHC molecules. Exposure to the allergen may be environmental or may involve administering the allergen to the subject. Allergic reactions may be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, it may be necessary to inhibit T cell mediated

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allergic responses locally or systemically by proper administration of a CTLA4-immunoglobulin fusion protein of the invention. For example, in one embodiment, a CTLA4-immunoglobulin fusion protein of the invention and an allergen are coadministered subcutaneously to an allergic subject.

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4. Virally Infected or Malignant T Cells: Inhibition of T cell activation through blockage of the interaction of a CTLA4 ligand with a receptor therefor on T cells may also be important therapeutically in viral infections of T cells. For example, in the acquired immune deficiency syndrome (AIDS), viral replication is stimulated by T cell activation. Blocking a CTLA4 ligand/receptor interaction, such as the interaction of B7-1 and/or B7-2 with CD28 and/or CTLA4 could lead to a lower level of viral replication and thereby ameliorate the course of AIDS. Surprisingly, HTLV-I infected T cells express B7-1 and B7-2. This expression may be important in the growth of HTLV-I infected T cells and the blockage of B7-1 function together with the function of B7-2 with a CTLA4-immunoglobulin fusion protein, possibly in conjunction with another blocking reagent (such as an anti-B7-2 blocking antibody or a CD28lg fusion protein) may slow the growth of HTLV-I induced leukemias. In addition, some tumor cells are responsive to cytokines and the inhibition of T cell activation and cytokine production could help to inhibit the growth of these types of cancer cells.

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5. Antigen-Specific T Cell Unresponsiveness: The methods of the invention for inhibiting T cell responses can essentially be applied to any antigen (e.g., protein) to clonally delete T cells responsive to that antigen in a subject. For example, in one study, administration of a CTLA4-IgG1 fusion protein to mice *in vivo* suppressed primary and secondary T cell-dependent antibody responses to antigen (Linsley P.S., et al. (1992) *Science* 257, 792-795). Thus, a subject treated with a molecule capable of inducing a T cell response can be treated with CTLA4-immunoglobulin fusion protein to inhibit T cell responses to the molecule. This basic approach has widespread application as an adjunct to therapies which utilize a potentially immunogenic molecule for therapeutic purposes. For example, an increasing number of therapeutic approaches utilize a proteinaceous molecule, such as an antibody, fusion protein or the like, for treatment of a clinical disorder. A limitation to the use of such molecules therapeutically is that they can elicit an immune response directed against the therapeutic molecule in the subject being treated (e.g., the efficacy of murine monoclonal antibodies in human subjects is hindered by the induction of an immune response against the antibodies in the human subject). Administration of a CTLA4-immunoglobulin fusion protein to inhibit antigen-

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specific T cell responses can be applied to these therapeutic situations to enable long term usage of the therapeutic molecule in the subject without elicitation of an immune response. For example, a therapeutic antibody (e.g., murine mAb) is administered to a subject (e.g., human), which typically activates T cells specific for the antibody in the subject. To inhibit the T cell response against the therapeutic antibody, the therapeutic antibody is administered to the subject together with a CTLA4-immunoglobulin fusion protein of the invention.

When used therapeutically, a CTLA4-immunoglobulin fusion protein of the invention can be used alone or in conjunction with one or more other reagents that influence immune responses. A CTLA4-immunoglobulin fusion protein and another immunomodulating reagent can be combined as a single composition or administered separately (simultaneously or sequentially) to downregulate T cell mediated immune responses in a subject. Examples of other immunomodulating reagents include blocking antibodies, e.g., against B7-1, B7-2 or other B cell surface antigens or cytokines, other fusion proteins, e.g., CD28Ig, or immunosuppressive drugs, e.g., cyclosporine A or FK506.

The CTLA4-immunoglobulin fusion proteins of the invention may also be useful in the construction of therapeutic agents which block immune cell function by destruction of the cell. For example, by linking a CTLA4-immunoglobulin fusion protein to a toxin such as ricin or diphtheria toxin, an agent capable of preventing immune cell activation would be made. Infusion of one or a combination of immunotoxins into a patient would result in the death of immune cells, particularly of activated B cells that express higher amounts of B7-1 and/or B7-2.

B. Screening Assays

Another application of the CTLA4-immunoglobulin fusion proteins of the invention is the use the protein in screening assays to discover as yet undefined molecules which inhibit an interaction between CTLA4 and a CTLA4 ligand, such as B7-1 or B7-2. For example, the CTLA4-immunoglobulin fusion protein can be used in a solid-phase binding assay in which panels of molecules are tested. In one embodiment, the screening method of the invention involves contacting a CTLA4-immunoglobulin fusion protein of the invention with a CTLA4 ligand and a molecule to be tested. Either the CTLA4-immunoglobulin fusion protein or the CTLA4 ligand is labeled with a detectable substance, such as a radiolabel or biotin, which allows for detection and quantitation of the amount of binding of CTLA4-immunoglobulin to the CTLA4 ligand. After allowing CTLA4-immunoglobulin and the CTLA4 ligand to

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interact in the presence of the molecule to be tested, unbound labeled CTLA4-immunoglobulin fusion protein or unbound labeled CTLA4 ligand is removed and the amount of CTLA4-immunoglobulin fusion protein bound to the CTLA4 ligand is determined. A reduced amount of binding of CTLA4-immunoglobulin fusion protein to the CTLA4 ligand in the presence of the molecule tested relative to the amount of binding in the absence of the molecule is indicative of an ability of the molecule to inhibit binding of CTLA4 to the CTLA4 ligand. Suitable CTLA4 ligands for use in the screening assay include B7-1 or B7-2 (e.g., B7-1Ig or B7-2Ig fusion proteins can be used). Preferably, either the unlabeled CTLA4-immunoglobulin fusion protein or the unlabeled CTLA4 ligand is immobilized on a solid phase support, such as a polystyrene plate or bead, to facilitate removal of the unbound labeled protein from the bound labeled protein.

C. Antibody Production

The CTLA4-immunoglobulin fusion proteins produced from the nucleic acid molecules of the invention can also be used to produce antibodies specifically reactive with the fusion protein and in particular with the CTLA4 moiety thereof (i.e., anti-CTLA4 antibodies). For example, by immunization with a CTLA4-immunoglobulin fusion protein, anti-CTLA4 polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the fusion protein which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a protein include conjugation to carriers or other techniques well known in the art. For example, the protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. An ELISA or other immunoassay which distinguishes antibodies reactive with the CTLA4 portion of the fusion protein from those which react with the IgC region are preferred (e.g., the extracellular domain of CTLA4 alone can be used in a standard ELISA to detect anti-CTLA4 antibodies).

Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. Examples include the hybridoma technique originally developed by Kohler and Milstein

(*Nature* (1975) 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., *Immunol. Today* (1983) 4:72), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. *Monoclonal Antibodies in Cancer Therapy* (1985) (Allen R. Bliss, Inc., pages 77-96), and screening of
5 combinatorial antibody libraries (Huse et al., *Science* (1989) 246:1275). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with CTLA4 and monoclonal antibodies isolated.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with a CTLA4-immunoglobulin fusion protein as described
10 herein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The term "antibody" is further intended to include bispecific and chimeric
15 molecules having an anti-CTLA4-immunoglobulin fusion protein portion, chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region, and humanized antibodies in which parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human
20 origin. Techniques for preparing chimeric or humanized antibodies are well known in the art (see e.g., Morrison et al., *Proc. Natl. Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B, Teng et
25 al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today*, 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982); PCT Publication WO92/06193 and EP 0239400). Another method of generating specific antibodies, or antibody fragments, reactive against a CTLA4-immunoglobulin fusion protein is to screen expression libraries encoding immunoglobulin genes, or portions thereof,
30 expressed in bacteria with a fusion protein produced from the nucleic acid molecules of the invention. For example, complete Fab fragments, V_H regions, F_V regions and single chain F_V regions can be expressed in bacteria using phage expression libraries. See for example Ward et al., *Nature*, 341: 544-546: (1989); Huse et al., *Science*, 246: 1275-1281 (1989); and McCafferty et al., *Nature*, 348: 552-554 (1990). Screening such
35 libraries with, for example, a CTLA4-immunoglobulin fusion protein can identify

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immunoglobulin fragments reactive with the protein, in particular the CTLA4 portion thereof.

5 An anti-CTLA4 antibody generated using the CTLA4-immunoglobulin fusion proteins described herein can be used therapeutically to inhibit immune cell activation through blocking receptor:ligand interactions necessary for stimulation of the cell. These so-called "blocking antibodies" can be identified by their ability to inhibit T cell proliferation and/or cytokine production when added to an *in vitro* costimulation assay as described herein. The ability of blocking antibodies to inhibit T cell functions may result in immunosuppression and/or tolerance when these antibodies are administered *in*
10 *vivo*.

D. Protein Purification

The CTLA4-immunoglobulin fusion proteins of the invention can be used to isolate CTLA4 ligands from cell extracts or other preparations. For example, a CTLA4-
15 immunoglobulin fusion protein can be used to immunoprecipitate B7-1, B7-2 or an as yet unknown CTLA4 ligand from a whole cell, cytosolic or membrane protein extract prepared from B cells or other antigen presenting cell using standard techniques. Additionally, anti-CTLA4 polyclonal or monoclonal antibodies prepared as described herein using a CTLA4-immunoglobulin fusion protein as an immunogen can be used to
20 isolate the native CTLA4 antigen from cells. For example, antibodies reactive with the CTLA4 portion of the CTLA4-immunoglobulin fusion protein can be used to isolate the naturally-occurring or native form of CTLA4 from activated T lymphocytes by immunoaffinity chromatography using standard techniques.

25 This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference.

30 **EXAMPLE 1: Preparation of CTLA4-immunoglobulin Fusion Proteins with Reduced Effector Function**

The extracellular portion of the T cell surface receptor CTLA4 was prepared as a fusion protein coupled to an immunoglobulin constant region. The immunoglobulin constant region was genetically modified to reduce or eliminate effector activity inherent
35 in the immunoglobulin structure. Briefly, DNA encoding the extracellular portion of CTLA4 was joined to DNA encoding the hinge, CH2 and CH3 regions of human IgC γ 1 or IgC γ 4 modified by directed mutagenesis. This was accomplished as follows:

Preparation of Gene Fusions

DNA fragments corresponding to the DNA sequences of interest were prepared by polymerase chain reaction (PCR) using primer pairs described below. In general, PCR reactions were prepared in 100 µl final volume composed of Taq polymerase buffer (Gene Amp PCR Kit, Perkin-Elmer/Cetus, Norwalk, CT) containing primers (1 µM each), dNTPs (200 µM each), 1 ng of template DNA, and Taq polymerase (Saiki, R.K., et al. (1988) *Science* 239:487-491). PCR DNA amplifications were run on a thermocycler (Ericomp, San Diego, CA) for 25 to 30 cycles each composed of a denaturation step (1 minute at 94 °C), a renaturation step (30 seconds at 54 °C), and a chain elongation step (1 minute at 72 °C).

To create gene fusions encoding hybrid proteins, "zip up" PCR was used. This procedure is diagrammed schematically in Figure 1. A first set of forward (A) and reverse (C) primers was used to amplify the first gene segment of the gene fusion. A second set of forward (B) and reverse (D) primers was used to amplify the second gene segment of the gene fusion. Primers B and C were designed such that they contained complimentary sequences capable of annealing. The PCR products amplified by primers A + C and B + D are combined, annealed and extended ("zipped up"). The full-length gene fusion was then amplified in a third PCR reaction using the "zip up" fragment as the template and primers A and D as the forward and reverse primers, respectively.

The structure of each CTLA4 genetic fusion consisted of a signal sequence, to facilitate secretion, coupled to the extracellular domain of CTLA4 and the hinge, CH2 and CH3 domains of human IgCγ1 or IgCγ4. The IgC γ1 and IgC γ4 sequences were modified to contain nucleotide changes within the hinge region to replace cysteine residues available for disulfide bond formation and to contain nucleotide changes in the CH2 domain to replace amino acids thought to be required for IgC binding to Fc receptors and complement activation. The hinge region and CH2 domain amino acid mutations introduced into IgCγ1 and IgCγ4 are illustrated in Figure 2A and 2B, respectively.

A. Construction of CTLA4-Ig Fusion Genes

1. Preparation of the Signal Sequence Gene Segment

PCR amplification was used to generate an immunoglobulin signal sequence suitable for secretion of the CTLA4-Ig fusion protein from mammalian cells. The Ig signal sequence was prepared from a plasmid containing the murine IgG heavy chain

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gene (described in Orlandi, R., et al. (1989) *Proc. Natl. Acad. Sci. USA* **86**:3833-3837) using the oligonucleotide 5'CATTCTAGAACCTCGACAAGCTTGAGATCACAG-TTCTCTCTAC-3' (SEQ ID NO: 1) as the forward primer and the oligonucleotide 5'CAGCAGGCTGGGCCACGTGCATT GCGGAGTGGACACCTGTGGAGAG-3' (SEQ ID NO: 2) as the reverse PCR primer. The forward PCR primer (SEQ ID NO: 1) contains recognition sequences for restriction enzymes XbaI and HindIII and is homologous to sequences 5' to the initiating methionine of the Ig signal sequence. The reverse PCR primer (SEQ ID NO: 2) is composed of sequences derived from the 5' end of the extracellular domain of CTLA4 and the 3' end of the Ig signal sequence. PCR amplification of the murine Ig signal template DNA using these primers resulted in a 233 bp product which is composed of XbaI and HindIII restriction sites followed by the sequence of the Ig signal region fused to the first 25 nt of the coding sequence of the extracellular domain of CTLA4. The junction between the signal sequence and CTLA4 is such that protein translation beginning at the signal sequence will continue into and through CTLA4 in the correct reading frame.

2. Preparation of the CTLA4 Gene Segment

The extracellular domain of the CTLA4 gene was prepared by PCR amplification of plasmid pHCTLA4. This plasmid contained the sequences corresponding to the human CTLA4 cDNA (see Darivach, P., et al., (1988) *Eur. J. Immunol.* **18**:1901-1905; Harper, K., et al., (1991) *J. Immunol.* **147**: 1047-1044) inserted into the multiple cloning site of vector pBluescript (Stratagene, La Jolla, CA) and served as the template for a PCR amplification using the oligonucleotide 5'-CTCTCCACAGGTGTCCACT-CCGCAATGCACGTGG CCCAGCCTGCTG-3' (SEQ ID NO: 3) as the forward PCR primer and the oligonucleotide 5'-TGTGTGTGGAATTCTCATTACTGATCAGA-ATCTGGGCACGGTTCTG-3' (SEQ ID NO: 4) as the reverse PCR primer. The forward PCR primer (SEQ ID NO: 3) was composed of sequences derived from the 3' end of the Ig signal sequence and the 5' end of the extracellular domain of CTLA4. This PCR primer is the complementary to murine Ig signal reverse PCR primer (SEQ ID NO: 2). The reverse PCR primer (SEQ ID NO: 4) was homologous to the 3' end of the extracellular domain of CTLA4, added a BclI restriction site and an additional G nucleotide at the end of the extracellular domain. This created a unique BclI restriction site and added a glutamine codon to the C-terminus of the extracellular domain. The final PCR product was 413 bp.

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3. Fusion of the Immunoglobulin Signal Sequence and CTLA4 Gene Segments

The PCR fragments containing the signal and CTLA4 sequences were joined together via a third PCR reaction. Both PCR fragments (1 ng each) were mixed together along with the Ig signal forward PCR primer (SEQ ID NO: 1) and the CTLA4 reverse PCR primer (SEQ ID NO: 4) and PCR amplified as described. In this reaction, the 3' end of the Ig signal fragment hybridizes with the 5' end of the CTLA4 fragment and the two strands are extended to yield a full length 600 bp fragment. Subsequent PCR amplification of this fragment using forward (SEQ ID NO: 1) and reverse (SEQ ID NO: 4) yielded sufficient amounts of the signal-CTLA4 gene fusion fragment for cloning. This fragment contains a 5' XbaI and a 3' BclI restriction sites flanking the Ig signal-CTLA4 gene fusion segment for subsequent cloning.

4. Cloning of Immunoglobulin Constant Domain Gene Segments

Plasmid pSP72IgG1 was prepared by cloning the 2000 bp segment of human IgG1 heavy chain genomic DNA (Ellison, J.W., et al., (1982) *Nucl. Acids. Res.* 10:4071-4079) into the multiple cloning site of cloning vector pSP72 (Promega, Madison, WI). Plasmid pSP72IgG1 contained genomic DNA encoding the CH1, hinge, CH2 and CH3 domain of the heavy chain human IgG1 gene. PCR primers designed to amplify the hinge-CH2-CH3 portion of the heavy chain along with the intervening genomic DNA were prepared as follows. The forward PCR primer, 5'-GCATTTTAAGCTTTTCCTGATCAGGAGCC CAAATCTTCTGACAAAA-CTCACACATCTCCACCGTCTCCAGGTAAGCC-3' (SEQ ID NO: 5), contained HindIII and BclI restriction sites and was homologous to the hinge domain sequence except for five nucleotide substitutions which changed the three cysteine residues to serines. The reverse PCR primer, 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO: 6), was identical to the commercially available T7 primer (Promega, Madison, WI). Amplification with these primers yielded a 1050 bp fragment bounded on the 5' end by HindIII and BclI restriction sites and on the 3' end by BamHI, SmaI, KpnI, SacI, EcoRI, ClaI, EcoR5 and BglII restriction sites. This fragment contained the IgG1 hinge domain in which the three cysteine codons had been replaced by serine codons followed by an intron, the CH2 domain, an intron, the CH3 domain and additional 3' sequences. After PCR amplification, the DNA fragment was digested with HindIII and EcoRI and cloned into expression vector pNRDSH (Repligen; Cambridge, MA (diagrammed in Figure 3)) digested with the same restriction enzymes. This created plasmid pNRDSH/IgG1.

A similar PCR based strategy was used to clone the hinge-CH2-CH3 domains of human IgC γ 4 constant regions. A plasmid, p428D (Medical Research Council, London, England) containing the complete IgC γ 4 heavy chain genomic sequence (Ellison, J., et al., (1981) *DNA* 1: 11-18) was used as a template for PCR amplification using

5 oligonucleotide 5'GAGCATTTTCCTGATCAGGAGTCCAAATATGGTCCCCC-
ACCCC ATCATCCCCAGGTAAGCCAACCC-3' (SEQ ID NO: 7) as the forward PCR
primer and oligonucleotide 5'GCAGAGGAATTCGAGCTCGGTACCCGGGGATCC-
CCAGTGTGGGGACAGTGGGACCCGCTCTGCCTCCC-3' (SEQ ID NO: 8) as the
reverse PCR primer. The forward PCR primer (SEQ ID NO: 7) contains a BclI
10 restriction site followed by the coding sequence for the hinge domain of IgC γ 4. Nucleotide substitutions have been made in the hinge region to replace the cysteines
residues with serines. The reverse PCR primer (SEQ ID NO: 8) contains a PspAI
restriction site. PCR amplification with these primers results in a 1179 bp DNA
fragment. The PCR product was digested with BclI and PspAI and ligated to
15 pNRDSH/IgG1 digested with the same restriction enzymes to yield plasmid
pNRDSH/IgG4. In this reaction, the IgC γ 4 domain replaced the IgC γ 1 domain present in
pNRDSH/IgG1.

5. Modification of Immunoglobulin Constant Domain Gene Segments

20 Modification of the CH2 domain in IgC to replace amino acids thought to be
involved in binding to Fc receptor was accomplished as follows. Plasmid
pNRDSH/IgG1 served as template for modifications of the IgC γ 1 CH2 domain and
plasmid pNRDSH/IgG4 served as template for modifications of the IgC γ 4 CH2 domain.
Plasmid pNRDSH/IgG1 was PCR amplified using a forward PCR primer (SEQ ID NO:
25 5) and oligonucleotide 5'-GGGTTTT GGGGGAAGAGGAAGACTGACGGTGCC-
CCC TCGGCTTCAGGTGCTGAGGAAG-3' (SEQ ID NO: 9) as the reverse PCR
primer. The forward PCR primer (SEQ ID NO: 5) has been previously described and
the reverse PCR primer (SEQ ID NO: 9) was homologous to the amino terminal portion
of the CH2 domain of IgG1 except for five nucleotide substitutions designed to change
30 amino acids 234, 235, and 237 from Leu to Ala, Leu to Glu, and Gly to Ala, respectively
(Canfield, S. M. and Morrison, S. L. (1991) *J. Exp. Med.* 173: 1483-1491; see Figure
2A). Amplification with these PCR primers will yield a 239 bp DNA fragment
consisting of a modified hinge domain, an intron and modified portion of the CH2
domain.

35 Plasmid pNRDSH/IgG1 was also PCR amplified with the oligonucleotide 5'-
CATCTCTTCCTCAGCACCTGAAGCCGAGGGGGCACCCTCAGTCTTCCTCTTC

CC CC-3' (SEQ ID NO: 10) as the forward primer and oligonucleotide (SEQ ID NO: 6) as the reverse PCR primer. The forward PCR primer (SEQ ID NO: 10) is complementary to primer (SEQ ID NO: 9) and contains the five complementary nucleotide changes necessary for the CH2 amino acid replacements. The reverse PCR
 5 primer (SEQ ID NO: 6) has been previously described. Amplification with these primers yields a 875 bp fragment consisting of the modified portion of the CH2 domain, an intron, the CH3 domain, and 3' additional sequences.

The complete IgC γ 1 segment consisting of modified hinge domain, modified CH2 domain and CH3 domain was prepared by an additional PCR reaction. The
 10 purified products of the two PCR reactions above were mixed, denatured (95 °C, 1 minute) and then renatured (54 °C, 30 seconds) to allow complementary ends of the two fragments to anneal. The strands were filled in using dNTP and Taq polymerase and the entire fragment amplified using forward PCR primer (SEQ ID NO: 5) and reverse PCR primer (SEQ ID NO: 6). The resulting fragment of 1050 bp was purified, digested with
 15 HindIII and EcoRI and ligated to pNRDSH previously digested with the same restriction enzymes to yield plasmid pNRDSH/IgG1m.

Two amino acids at immunoglobulin positions 235 and 237 were changed from Leu to Glu and Gly to Ala, respectively, within the IgC γ 4 CH2 domain to eliminate Fc receptor binding (see Figure 2B). Plasmid pNRDSH/IgG4 was PCR amplified using the
 20 forward primer (SEQ ID NO: 7) and the oligonucleotide 5'CGCACGTGACCT CAGGGGTCCGGG AGATCATGAGAGTGTCTTGGGTTTTGGGGGAAC- AGGAAGACTGATG GTGCCCCCTCGAACTCAGGTGCTGAGG-3' (SEQ ID NO: 11) as the reverse primer. The forward primer has been previously described and the reverse primer was homologous to the amino terminal portion of the
 25 CH2 domain, except for three nucleotide substitutions designed to replace the amino acids described above. This primer also contained a PmlI restriction site for subsequent cloning. Amplification with these primers yields a 265 bp fragment composed of the modified hinge region, and intron, and the modified 5' portion of the CH2 domain.

Plasmid pNRDSH/IgG4 was also PCR amplified with the oligonucleotide
 30 5'-CCTCAGCACCTGAGTTCGAGGGGGCACCATCAGTCTTCCTGTTCCCCCCC AAAACCCAAGGAACTCTCATGATCTCCCGGACCCCTGAGGTCACGTGCG-3' (SEQ ID NO: 12) as the forward primer and oligonucleotide (SEQ ID NO: 8) as the reverse PCR primer. The forward PCR primer (SEQ ID NO: 12) is complementary to primer (SEQ ID NO: 11) and contains the three complementary nucleotide changes
 35 necessary for the CH2 amino acid replacements. The reverse PCR primer

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(SEQ ID NO: 8) has been previously described. Amplification with these primers yields a 1012 bp fragment consisting of the modified portion of the CH2 domain, an intron, the CH3 domain, and 3' additional sequences.

5 The complete IgC γ 4 segment consisting of modified hinge domain, modified CH2 domain and CH3 domain was prepared by an additional PCR reaction. The purified products of the two PCR reactions above were mixed, denatured (95 °C, 1 minute) and then renatured (54 °C, 30 seconds) to allow complementary ends of the two fragments to anneal. The strands were filled in using dNTP and Taq polymerase and the entire fragment amplified using forward PCR primer (SEQ ID NO: 7) and reverse PCR
10 primer (SEQ ID NO: 8). The resulting fragment of 1179 bp was purified, digested with BclI and PspAI and ligated to pNRDSH previously digested with the same restriction enzymes to yield plasmid pNRDSH/IgG4m.

6. Assembly of CTLA4-Immunoglobulin Fusion Genes

15 The PCR fragment corresponding to the Ig signal-CTLA4 gene fusion prepared as described above (sections 1-3) was digested with HindIII and BclI restriction enzymes and ligated to pNRDSH/IgG1, pNRDSH/IgG1m, pNRDSH/IgG4, and pNRDSH/IgG4m previously digested with the same restriction enzymes to create expression plasmids in which the signal-CTLA4-IgG gene fusion segment is placed
20 under the control of the CMV promoter. The ligated plasmids were transformed into *E. coli* JM109 using CaCl₂ competent cells and transformants were selected on L-agar containing ampicillin (50 µg/ml; as described in *Molecular Cloning: A Laboratory Manual* (1982) Eds. Maniatis, T., Fritsch, E. E., and Sambrook, J. Cold Spring Harbor Laboratory). Plasmids isolated from the transformed *E. coli* were analyzed by
25 restriction enzyme digestion. Plasmids with the expected restriction pattern were sequenced to verify all portions of the signal-CTLA4-IgG gene fusion segments. The final plasmids were named pNRDSH/sigCTLA4-IgG1, pNRDSH/sigCTLA4-IgG1m, pNRDSH/sigCTLA4-IgG4 and pNRDSH/sigCTLA4-IgG4m. The signal-CTLA4-IgG gene fusion segments from each of these constructs were also transferred to the pEE12
30 expression vector (*Biotechnology* (1992) 10:169-175).

The nucleotide and predicted amino acid sequences of the signal-CTLA4-IgG gene fusion segments are shown in the Sequence Listing as follows: sigCTLA4-IgGm- SEQ ID NOS: 23 and 24, sigCTLA4-IgG4- SEQ ID NOS: 25 and 26 and sigCTLA4-IgG4m- SEQ ID NOS: 27 and 28.

35

B. Construction of a CTLA4Ab Fusion Gene

The extracellular domain of CTLA4 is an immunoglobulin superfamily member and is responsible for binding to its ligands B7-1 and B7-2. The replacement of the heavy and light chain variable domains of an antibody molecule with the extracellular domain of CTLA4 will result in an antibody-like protein which can bind specifically to B7-1, B7-2 and other CTLA4 ligands with high affinity. The construction of such a molecule using human IgG1 antibody heavy and light chains is described below.

1. Construction of the Heavy Chain Gene

The Ig signal sequence was prepared from template plasmid pSP72IgG1 by PCR amplification using oligonucleotide 5'CATTGCTTACCTCGACAAGCTTGAGATCAC AGTTCTCTCTAC-3' (SEQ ID NO: 13) as the forward PCR primer and oligonucleotide 5'-GGAGTGGACACCTGTGGAGAG-3' (SEQ ID NO: 14) as the reverse primer. The forward PCR primer (SEQ ID NO: 13) contains a HindIII restriction site and part of the 5' untranslated segment of the Ig signal domain. The reverse PCR primer (SEQ ID NO: 14) corresponds to the C-terminus of the natural Ig signal peptide. Amplification with these primers resulted in a 208 bp fragment encoding the entire Ig signal sequence.

The CTLA4 extracellular domain was prepared from plasmid phCTLA4, which contained the entire CTLA4 cDNA sequence, by PCR amplification using oligonucleotide 5'-CTCCACAGGTGTCCACTCCGCAATGCACGTGGCCCAGCC-3' (SEQ ID NO: 15) as the forward PCR primer and oligonucleotide 5'GAGGTTGTAAG-GACTCACCTGAAA TCTGGGCTCCGTTGC-3' (SEQ ID NO: 16) as the reverse primer. The forward primer (SEQ ID NO: 15) contained sequences homologous to the 5' end of the CTLA4 extracellular domain and to the 3' end of the Ig signal domain. The reverse primer (SEQ ID NO: 16) contained the 3' end of the CTLA4 extracellular domain and intervening sequences, including a splice acceptor site. Amplification with these primers yielded a 379 bp fragment containing the CTLA4 extracellular domain.

An intervening sequence DNA fragment derived from the intron between the antibody variable and constant (CH1) domains was prepared by PCR amplification using oligonucleotide 5'GCAACGGAGCCCAGATTTCAGGTGAGTCCTTACA-ACCTC-3' (SEQ ID NO: 17) as the forward PCR primer and oligonucleotide 5'GGCTAGATATCTCTAGACT ATAAATCTCTGGCCATGAAG-3' (SEQ ID NO: 18) as the reverse PCR primer. The forward PCR primer (SEQ ID NO: 17) contains intron sequence and is complementary to the 3' end of the extracellular domain of CTLA4 and is complimentary to the CTLA4 reverse PCR

primer (SEQ ID NO: 16). The reverse primer (SEQ ID NO: 18) contains intron sequences and an additional XbaI restriction site. Amplification with these primers yields a 197 bp fragment.

5 The PCR fragments encoding the Ig signal, CTLA4 extracellular domain and the intervening sequence were mixed, denatured and renatured to allow hybridization of complementary ends. The strands were filled in and the product amplified using forward (SEQ ID NO: 13) and reverse (SEQ ID NO: 18) PCR primers. The product was a 764 bp fragment which encoded the Ig signal, the CTLA4 extracellular domain, an intron sequence flanked by HindIII and XbaI restriction sites. This DNA fragment was
10 digested with HindIII and XbaI and ligated to pSP72IgG1, resulting in the CTLA4 extracellular domain being linked to a 5' Ig signal sequence and a 3' antibody CH1, hinge, CH2, and CH3 domains.

The nucleotide and predicted amino acid sequences of the assembled CTLA4-heavy chain are shown in SEQ ID NOS: 29 and 30, respectively.

15

2. Construction of the Light Chain Gene

The replacement of a human immunoglobulin antibody light chain variable domain (Hieter, P.A., et al., (1980) *Cell* 22:197) with the CTLA4 extracellular domain proceeded as follows. The Ig signal fragment was prepared as for the heavy chain
20 replacement, described above. The CTLA4 extracellular domain was prepared using a forward PCR primer (SEQ ID NO: 15) previously described and oligonucleotide 5'-GGCACTAGGTCTGACTCTAGAAA CTGAGGAAGCAAAGTTTAAATTCTACTC-ACGTTTAATCTGGGCTCCGTTGC-3' (SEQ ID NO: 19) as the reverse primer. The reverse primer contained sequences of the 3' end of the CTLA4 extracellular domain, a
25 splice receptor, and intervening sequence DNA containing an XbaI restriction site. The Ig signal fragment and the CTLA4 extracellular domain were joined by mixing the DNA fragment, denaturing, and renaturing to anneal their complementary ends. The strands were filled in and the fragment PCR amplified using forward (SEQ ID NO: 13) and reverse (SEQ ID NO: 19) PCR primers previously described. The resulting DNA
30 fragment was digested with the HindIII and XbaI and ligated to immunoglobulin light chain vector p α LYS17 digested with the same enzymes. The resulting plasmid pCTLA4kappa contains an Ig signal sequence, an intron, the CTLA4 extracellular domain, an intron, and the light chain (kappa) constant domain.

The nucleotide and predicted amino acid sequences of the assembled
35 CTLA4-light chain are shown in SEQ ID NOS: 31 and 32, respectively.

The DNA segments encoding the recombinant heavy and light chains were transferred to the pEE12 vector or the pNRDSH vector and stable NSO or CHO expression cell lines established as described below. CHO and NSO supernatants were assayed for the production of CTLA4 light chain and CTLA4 heavy chain fusion proteins by ELISA and binding to B7-1 was measured using CHO/hB7-1 expressing cells and FACS (as described in Example 2). It is also contemplated that the heavy and light chain constructs of the present invention be expressed in the same vector and host cells transfected in one step.

10 C. Expression of CTLA4 Fusion Proteins in CHO and NSO cells

The various CTLA4-immunoglobulin fusion proteins were expressed in CHO cells as follows. Briefly, 5×10^5 CHO-DG44 cells (subline of CHO-K1, available from ATCC) were transfected with 10 μ g of the appropriate expression plasmid (pNRDSH series) by the calcium phosphate method (described in *Molecular Cloning: A Laboratory Manual* (1982) Eds. Maniatis, T., Fritsch, E. E., and Sambrook, J. Cold Spring Harbor Laboratory) using a commercially available kit (5 Prime to 3' Prime Inc., Boulder, CO) according to the manufacturer's instructions. The transfected cells were allowed to recover in nonselective media (alpha MEM medium containing 10 % heat inactivate fetal bovine serum (FBS), Gibco/BRL, Gaithersburg, MD) for two days and then plated in selective media (alpha MEM minus nucleoside medium containing 10 % FBS and 550 μ g/ml G418; Gibco/BRL, Gaithersburg, MD). Individual subclones were obtained by dilution cloning in selective media. Culture media was assayed for the presence of secreted CTLA4-immunoglobulin by a standard ELISA designed to detect human IgG.

25 The various CTLA4-immunoglobulin and CTLA4Ab fusion proteins were expressed in NSO cells (Golfre, G. and Milstein C.P. (1981) *Methods Enzymol.* 73B: 3-46) as follows. Briefly, 10^7 NSO cells were transfected by electroporation (using a BioRad Gene Pulser, Hercules, CA) with 40 μ g of the appropriate expression plasmid (pEE12 series) previously linearized by digestion with Sall restriction endonuclease. The transfected cells were selected using DMEM media deficient in glutamine (Gibco/BRL, Gaithersburg MD). Individual subclones were isolated by dilution cloning in selective media. Culture media assayed for the presence of secreted CTLA4-Ig or CTLA4Ab fusion protein by a standard ELISA assay designed to detect human IgG.

30 As a representative example, transfection of either the pNRDSH/sigCTLA4-IgG4m and pEE12/sigCTLA4-IgG4m expression vector into CHO or NSO host cells

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resulted in selected subclones that secreted hCTLA4IgG4m fusion protein into culture supernatants at a concentration of 75-100 µg/ml.

D. Purification of CTLA4 Fusion Proteins

5 The CTLA4-Ig and CTLA4Ab fusion proteins are purified from the culture medium of transfected CHO or NSO cells as follows. Culture medium was concentrated 10 fold by ultra filtration (Ultrasette, Filtron Technology Corp., Northborough, MA) and batch bound overnight to immobilized protein A (IPA-300, Repligen Corp., Cambridge, MA). The protein-bound resin was poured into a chromatography column, washed with 10 column volumes of optimal binding buffer (1.5 M glycine, 3M NaCl, pH 8.9) and the bound CTLA4-Ig or CTLA4Ab was eluted by the addition of 0.1 M Na citrate, pH 3.0. Fractions were collected and neutralized with the addition of 1 M Tris base to pH of 7.0. The Abs_{280nm} was monitored for each fraction and peak fractions were analyzed by SDS-PAGE, followed by Coomassie Blue staining and Western blot analysis using an anti-CTLA4 polyclonal antiserum (described in Lindsten, T. et al. (1993) *J. Immunol.* 151:3489-3499). Fractions containing CTLA4-Ig or CTLA4Ab were pooled and dialyzed against 200 volumes of 0.5 x PBS overnight at 4 °C. The purified protein was assayed for binding to its ligand (B7-1 and/or B7-2) as described in Example 2.

20 **EXAMPLE 2: Characterization of CTLA4 Fusion Proteins**

The ability of the various CTLA4-Ig forms and CTLA4Ab to bind to their counter receptors B7-1 (Freeman, G.F., et al. (1988) *J. Immunol.* 143:2714-2722) and B7-2 (Freeman, G.F., et al., (1993) *Science* 262: 909-911) was demonstrated using the following assays.

25 A. Fluorescence Activated Cell Staining (FACS).

Purified preparations of the various recombinant CTLA4 forms were tested for their ability to bind to transfected COS cell transiently expressing hB7-1 or hB7-2 or transfected CHO cells stably expressing hB7-1 or hB7-2. The recombinant CTLA4 protein (10 µg/ml) was incubated with B7 expressing cells (2×10^6 cells) for 1 hr on ice in FACS wash solution (1 % bovine serum albumin in PBS). The cells were washed 3 times with FACS wash solution. The cell bound CTLA4 was detected by reaction with anti-human Ig-FITC (Dako Corporation, Carpinteria, CA) or protein A-FITC (Dako) for 30 minutes on ice in the dark. The cells were washed twice with FACS wash solution and then fixed in 1 % paraformaldehyde in PBS. The cells were analyzed for fluorescence intensity using a Becton Dickinson (San Jose, CA) FACS analyzer.

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Murine anti-human mAbs reactive with either hB7-1 or hB7-2 served as positive control reagents for the hB7-1 and hB7-2 receptor expressing cells. These mAbs were detected using goat anti-murine IgG-FITC (Dako corporation, Carpinteria, CA) and analyzed as above. Untransfected COS and CHO cells served as negative controls for each cell line.

- 5 The results of this experiment demonstrated that CTLA4 immunoglobulin fusion proteins bind to CHO cells transfected to express CTLA4 ligands.

B. Competitive Binding ELISA

- The ability of the various recombinant CTLA4 forms to bind to hB7-1 or hB7-2 was assessed in a competitive binding ELISA assay. This assay was established as follows. Purified recombinant hB7-Ig (50 μ l at 20 μ g/ml in PBS) was bound to a Costar EIA/RIA 96 well microtiter dish (Costar Corp, Cambridge MA, USA) overnight at room temperature. The wells were washed three times with 200 μ l of PBS and the unbound sites blocked by the addition of 1 % BSA in PBS (200 μ l/well) for 1 hour at room temperature. The wells were washed again as above. Biotinylated hCTLA4-IgG1 (prepared according to manufacturers instructions (Pierce, Rockford, IL) at 10 μ g/ml serially diluted in twofold steps to 15.6 ng/ml; 50 μ l/well) was added to each well and incubated for 2.5 hours at room temperature. The wells were washed again as above. The bound biotinylated hCTLA4IgG1 was detected by the addition of 50 μ l of a 1:2000 dilution of streptavidin-HRP (Pierce Chemical Co., Rockford, IL) for 30 minutes at room temperature. The wells were washed as above and 50 μ l of ABTS (Zymed, California) added and the developing blue color monitored at 405 nm after 30 min.

- The ability of the various forms of CTLA4 to compete with biotinylated CTLA4-IgG1 was assessed by mixing varying amounts of the competing protein with a quantity of biotinylated CTLA4-IgG1 shown to be non-saturating (i.e., 70 ng/ml; 1.5nM) and performing the binding assays as described above. A reduction in the signal (Abs_{405nm}) expected for biotinylated CTLA4-IgG1 indicated a competition for binding to plate-bound hB7-1 or hB7-2. A graphic representation of a typical binding assay illustrating the competition of biotinylated hCTLA4-IgG1 with hCTLA4-IgG1 (itself) or hCTLA4-IgG4m is shown in Figure 4A for binding to hB7-1 and Figure 4B for binding to hB7-2. The competition curves show that the mutant IgG4 form competes with hCTLA4-IgG1 for binding to B7-1 or B7-2 with the same binding kinetics as the unlabeled IgG1 form itself. Accordingly, mutation of the hinge region and CH2 domain of IgCy4 in the CTLA4 fusion protein as described herein does not detrimentally affect the ligand binding activity of the CTLA4 fusion protein.

C. SDS-PAGE and Western Blotting

The various CTLA4 forms were analyzed by SDS-PAGE followed by detection using Coomassie Blue staining or Western blotting. The CTLA4 proteins were separated on both reducing and non-reducing SDS-PAGE gels (9, 12, or 15 % gels with 5 % stacking gel) and stained with Coomassie Blue using standard methods. Protein size was estimated from comparison to commercial size standards (BioRad, Hercules, CA). Western blots were performed using standard procedures and Immobilon blotting membranes (Millipore, New Bedford, MA). The CTLA4 was detected using a polyclonal antisera raised in rabbit immunized with the extracellular domain of CTLA4 produced in *E. coli* (described in Lindsten, T. et al. (1993) *J. Immunol.* 151:3489-3499). The CTLA4 was visualized using [¹²⁵I]-protein A (Dupont NEN, Boston, MA) followed by autoradiography or using protein A-HRP. The results indicated the presence of an immunoreactive band at approximately 50 kD.

15 D. Measurement of Fc Receptor Binding

The binding of the various CTLA4-Ig forms and CTLA4Ab to Fc receptors was assessed by using a competitive binding assay as described in Alegre, M.-L., et al., (1992) *J. Immunol.* 148:3461-3468. Human cell line U937 was used as a source of the FcRI and FcRII receptors (Looney, R.J., et al., (1986) *J. Immunol.* 136:1641). U937 cells were grown with 500 U/ml IFN- γ to upregulate expression of FcRI. The U937 cells were used at a concentration of 6.25×10^6 cells/ml. Preparations of unlabeled CTLA4-IgG1, CTLA4-IgG4 and human IgG1 were serially diluted to a concentration of 2×10^{-10} M. To each serial dilution, a fixed amount of ¹²⁵I-labeled protein (e.g., CTLA4-IgG1, CTLA4-IgG4 or human IgG1) was added. The U937 cells were then added to the mixture and incubated for three hours. The cells were separated from unbound labeled and unlabeled protein by centrifugation through silicone oil for one minute at 14000 x g. The tips of the tubes with the pelleted cells were then cut off and analyzed in a gamma counter. Maximal binding of labeled protein to U937 cells was determined in the absence of unlabeled competitor protein. Percent specific activity represents the percentage of labeled protein bound in the presence of unlabeled competitor protein relative to maximal binding. Figure 5A graphically illustrates the amount of labeled CTLA4-IgG1 bound to U937 cells (expressed in counts per minute) in the presence of unlabeled CTLA4-IgG1 or CTLA4-IgG4. Unlabeled CTLA4-IgG1 was able to compete with labeled CTLA4-IgG for binding to FcRI on U937 cells (i.e., the amount of bound labeled protein was reduced), whereas unlabeled CTLA4-IgG4 did not compete for binding. Figure 5B graphically illustrates the percent specific activity

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of labeled human IgG1, CTLA4-IgG1 and CTLA4-IgG4 being competed with themselves (unlabeled). The IC₅₀ for human IgG1 was approximately 7.5×10^{-8} M. The IC₅₀ for CTLA4-IgG1 was approximately 7×10^{-8} M. An IC₅₀ for CTLA4-IgG4 could not be determined because this protein did not bind to the FcR1. These results demonstrate that use of an IgG4 constant region in a CTLA4-Ig fusion protein essentially eliminates the ability of the fusion protein to bind to Fc receptors.

E. Measurement of Complement Activation

CTLA4-immunoglobulin forms were tested in a ligand-specific assay for complement activation. CHO cells expressing hB7-1 on their surface were grown to confluence in tissue culture dishes. After washing away serum and medium, the cells were exposed to BCECF/AM ([2',7-bis-(carboxyethyl)-5,(6')-carboxylfluorescein acetoxymethyl)-ester] (Calbiochem, La Jolla, CA) a fluorescent dye that irreversibly loads into the cells. The cells (5×10^5) were then incubated with hCTLA4-immunoglobulin fusion proteins or a monoclonal antibody specific for hB7-1 (4B2). Unbound protein was washed away and a complement source was added and allowed to react with the cells for 30 minutes. Complement sources tested included guinea pig complement and human serum (as a source of human complement). After incubation with the complement source, lysis was measured by monitoring the release of the fluorescent dye from the cells using a fluorometer. Controls included parallel experiments with hB7-1 negative CHO cells. Identical cultures were also tested for their ability to bind the hCTLA4 forms under similar assay conditions. Additionally, to distinguish a lack of an ability to activate complement from a lack of an ability to bind B7-1, an ELISA-type assay of CTLA4 binding to CHO-B7-1 cells was performed as a control (described further below).

The results of typical complement activation assays are shown in Figures 6A-C. Figure 6A graphically illustrates guinea pig complement-mediated lysis of CHO-B7-1 cells by CTLA4-IgG1, CTLA4-IgG4m and the anti-B7-1 monoclonal antibody 4B2. hCTLA4-IgG1 reproducibly activated guinea pig complement as well or better than the 4B2 mAb. The hCTLA4-IgG4m did not activate complement in this assay, even at concentration 100-fold higher than that needed for CTLA4-IgG1. The results were confirmed by repeating the work with human serum as the complement source, shown in Figure 6B. Human complement produced a higher percentage lysis than the guinea pig complement, however, otherwise the results were the same, with the hCTLA4-IgG4m exhibiting a markedly reduced ability to activate complement in comparison to CTLA4-IgG1. The effect of the CTLA4 fusion proteins on complement activation is specific for

the B7-1 ligand, as untransfected CHO cells were not substrates for complement activation by any of the proteins tested, illustrated in Figure 6C (using guinea pig complement as the complement source).

In order to verify that the hCTLA4-IgG4m form was still able to bind to membrane bound hB7-1, an experiment was performed by a similar method as for the complement activation study. Antibody or hCTLA4 forms were bound to washed CHO-B7-1 cells under conditions identical to those used in the complement activation studies except that instead of adding complement in the final step, an HRP-conjugated anti-Ig Fc (Calbiochem, La Jolla, CA) was used. Bound HRP was detected by washing the cells, adding ABTS substrate and measuring absorbance at 405 nm (as described above for the competition ELISA assay). The results are shown graphically in Figure 7. All three B7-1 specific proteins (mAb 4B2, hCTLA4-IgG1 and hCTLA4-IgG4m) bound to the cells. The corresponding experiment using untransfected CHO cells showed no binding of the proteins to the cells. The difference in the maximal O.D. signals for the different proteins is likely due to the different affinities of the forms of Fc regions for the HRP-conjugated secondary antibodies.

F. Inhibition of T Cell Proliferation

The ability of the CTLA4-Ig forms and CTLA4Ab to inhibit the proliferation of T cells in a costimulation proliferation assay was measured. CD4⁺ T cells are prepared from human blood by density gradient centrifugation on Ficoll-Hypaque (Sigma, St. Louis, MO). Monocytes were removed by adherence to plastic and the CD4⁺ cells further enriched by removal of residual monocytes, B cells, NK cells and CD8⁺ T cells by lysis with complement and mAbs (anti-CD14, antiCD11b, anti-CD20, anti-CD16 and anti-CD8) or by negative selection using the same immunomagnetic beads (Advanced Magnetics, Cambridge, MA) (as described in Boussioutis, V.A., et al., (1993) *J. Exp. Med.* 178:1758-1763). CD4⁺ T cells (10⁵) were cultured in the presence of immobilized anti-CD3 mAb (coated at 1 ug/well, overnight) and CHO cells expressing hB7-1 or hB7-2 (2x10⁴) in a microtiter plate with or without one of the CTLA4 forms and incubated for 3 days. Thymidine incorporation as a measure of mitogenic activity was assessed after overnight incubation in the presence of [³H] thymidine (Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci USA* 88:6575-6579). Inhibition was calculated as a percent of proliferation in control cultures. The data show that both the CTLA4IgG1 and CTLA4Ig4m performed well, inhibiting T cell proliferation to the same extent when used in equivalent amounts, i.e. the two compounds were indistinguishable in potency.

G. Pharmacokinetic Studies

The effect of mutating the IgG4 heavy chain, as described herein, on the pharmacokinetics of a CTLA4Ig in rats was examined. Pharmacokinetics were performed on two CTLA4Igs differing only in their heavy chain constant domains, where one form contained the wild type human IgG1 Ig (referred to as hCTLA4IgG1) and the second antibody contained the mutated version of human IgG4 (referred to as hCTLA4IgG4m). Two Sprague-Dawley male rats weighing 0.3-0.4 kg were used for each protein. The CTLA4Ig forms were infused at a dose of 2 mg/kg via a Teflon angiocath which was placed in the marginal ear vein. Two control animals received an infusion of PBS ($\text{Ca}^{++}\text{Mg}^{++}$ free) in the same manner. Blood samples were drawn at 0, 15, 30, 60, 90, 360, 480 minutes, 24, 36, 48 hours, 7, 14 and 28 days. The concentration of free antibody in heparinized plasma was determined by a standard ELISA. Antibody clearance rates were determined. α and β $t_{1/2}$ values were calculated using the P-Fit subroutine of the BIOSOFT Fig-p figure processor/parameter fitter. The results are shown below:

hCTLA4IgG1

α $t_{1/2}$ = 4.2 min

β $t_{1/2}$ = 288 min

hCTLA4IgG4m

α $t_{1/2}$ = 16.6 min

β $t_{1/2}$ = 214.2 min

Both CTLA4IgG1 and CTLA4IgG4m have similar clearance rates, with a rapid (4-16 min) α phase and a more prolonged (214-288 min) β phase indicating a serum half life of approximately 4 hours.

EXAMPLE 3: Preparation of *E. coli*-Expressed Human CTLA4

A. Intracellular Expression of CTLA4 in *E. coli*

1. Cloning and Expression of CTLA4 Extracellular Domain

The extracellular domain of CTLA4 was expressed in *E. coli* after cloning into expression vector pETCm11a. This vector was derived from expression vector pET-11a (Novagen Inc., Madison WI) by cloning a chloramphenicol resistance gene cassette into

the *ScaI* restriction site within the ampicillin resistance gene. The extracellular domain of CTLA4 was prepared from plasmid phCTLA4 by PCR amplification using oligonucleotide 5'GCAGAGAGACAT ATGGCAATGCACGTGGCCCAGCCTG-CTGTGG-3' (SEQ ID NO: 20) as forward primer and oligonucleotide 5'-GCAGAG-AGAGGATCCTCAGTCAGTTAGT CAGAATCTGGGCACGGTTCTGG-3' (SEQ ID NO: 21) as reverse primer. The forward PCR primer (SEQ ID NO: 20) contains an *NdeI* restriction site in which the ATG sequence in the *NdeI* restriction site is followed immediately by the codon for the first amino acid of mature CTLA4 (Dariavach, P., et al. (1988) *Eur. J. Immunol.* **18**:1901). The reverse PCR primer (SEQ ID NO: 21) contains a *BamHI* restriction site preceded by translation stop codons in all three reading frames preceded by the last amino acid just prior to the CTLA4 transmembrane domain. PCR amplification with these primer yields a 416 bp fragment bounded by *NdeI* and *BamHI* restriction sites which contains DNA sequences encoding the extracellular domain of CTLA4 preceded by a methionine codon. The PCR product was digested with *NdeI* plus *BamHI* and ligated to expression vector pETCm11a digested with the same restriction enzymes.

The ligated DNA was transfected into *E. coli* strains BL21, HMS174, RGN714 and RGN715 containing the lambda DE3 helper phage by standard techniques. Transformants were selected in L-agar containing chloramphenicol at 50 ug/ml. Individual transformants were selected and tested for CTLA4 expression after induction by treatment of cells with 0.5 mM IPTG. Whole cell extracts were analyzed on SDS-PAGE gel followed by Coomassie Blue staining and Western blot analysis. The majority of the CTLA4 protein in these cells was found in inclusion bodies.

25 2. Purification of CTLA4 from Inclusion Bodies

Recombinant CTLA4 was recovered from cell pellets by treating the washed cells in lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM PMSF, 5 mM EDTA, 0.5% Triton X-100, and lysozyme at 0.3 mg/ml) followed by sonication. The inclusion bodies were recovered by centrifugation at 20,000 x g and solubilized by treatment with solubilization buffer (50 mM Tris-HCl pH8.0, 8 M urea, 50 mM 2-mercaptoethanol (2-ME)). The solubilization was assisted by mixing for two hours at room temperature. The soluble fraction contained CTLA4. The CTLA4 was purified by chromatography on S-sepharose (Pharmacia, Piscataway, NJ) as follows. The CTLA4 containing supernatant was adjusted to pH 3.4 by the addition of glacial acetic and applied to a S-sepharose column equilibrated in column buffer (100 mM Na-acetate, pH6.5, 8 M urea, 50 mM 2-ME, and 5 mM EDTA). The column was washed with column buffer

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and the bound CTLA4 eluted with a linear salt gradient (NaCl, 0 to 1 M) prepared in column buffer. Peak fractions exhibiting high Abs_{280nm} values were pooled and dialyzed against dialysis buffer (100 mM Tris-HCl, pH8.0, 8 M urea, 50 mM 2-ME, 5 mM EDTA). Remaining contaminating proteins were eliminated by chromatography on a Sephacryl S-100 (Pharmacia, Piscataway, NJ) sizing column. The resulting preparation was greater than 95 % pure CTLA4 as estimated by SDS-PAGE followed by Coomassie Blue staining and Western blot analysis. Since the estimated size of monomeric recombinant CTLA4 produced in *E. coli* was approximately 15 kDa, all steps of the purification protocol were tested for the presence of a 15 kDa protein by SDS-PAGE and the presence of CTLA4 verified by Western blotting.

3. Refolding of Denatured CTLA4

The CTLA4 protein purified from inclusion bodies is fully reduced and denatured and must be properly refolded in a physiological buffer, with intact disulfide bridges, to be in "active" form (i.e., able to bind hB7-1). To avoid solubility problems a step gradient dialysis procedure was used to remove urea, detergents and reductants. The most successful refolding was obtained when the secondary and tertiary protein structure was encouraged first, by gradient dialysis, removing all urea and detergent while in the presence of the reductant DTT. Subsequent slow removal of the DTT appeared to reduce the number of random intradisulfide bonds. As a control, a sample of CTLA4 was dialyzed directly from gel filtration buffer to PBS.

The success of refolding was estimated by immunoprecipitation. 5µg of hB7-1-Ig, bound to protein A resin, was used to pull down active CTLA4 from a 10 µg aliquot of each refolding trial. Precipitated protein was run on a reducing SDS-PAGE, transferred to an Immobilon membrane (Millipore, New Bedford, MA) and probed with polyclonal antisera to CTLA4 (antisera 1438, described in Lindsten, T. et al. (1993) *J. Immunol.* 151:3489-3499). The relative amount of protein detected at 15 kDa was indicative of the success of the refolding process. Refolding was also evaluated by assaying CTLA4 binding activity in a competition ELISA as described in Example 2. A successful refolding consisted of approximately 5 % active protein, or about 2 mg of active protein from a 1 L bacterial culture.

B. Preparation of Secreted CTLA4 from *E. coli*

A secreted form of CTLA4 was prepared from *E. coli* as follows. The extracellular domain of CTLA4 was joined to the pelB signal sequence (Lei. S.-P., et al.,

(1987) *J. Bacteriol.* 169: 4379-4383) by PCR using plasmid phCTLA4 as template and oligonucleotide 5'GGCACTAGTCATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACT CGCTGCCCAACCAGCGATGGCCGAGCAATGCACGTGGCCAGCCTGCTGTGG-3' (SEQ ID NO: 20) as the forward primer and a reverse primer (SEQ ID NO: 21) previously described. The forward PCR primer 5'-GGCACTAGTCATGAAATACCTATTGCCT ACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCGAGCAATGCACGTGGCCAGCCTGCTGTGG-3' (SEQ ID NO: 22) contains a unique BspHI restriction site, the complete pelB signal sequence and the 5' end of the extracellular domain of CTLA4.

The reverse PCR primer (SEQ ID NO: 21) contains a unique BamHI restriction site preceded by translational stop codons in all three reading frames preceded by the last amino acid before the transmembrane domain of CTLA4. PCR amplification with these primers yielded a 480 bp fragment bounded by unique BspHI and BamHI restriction sites encoding the pelB signal sequence joined to the CTLA4 extracellular domain.

After PCR amplification, the DNA fragment was digested with BspHI and BamHI and ligated to expression vector pTrc99A (Pharmacia, Piscataway, New Jersey) previously digested with NcoI and BamHI. This resulted in a plasmid in which the expression of the pelB-CTLA4 protein was driven by the pTrc promoter present in the pTrc99A expression vector. *E. coli* host strains transformed with the ligated DNA were selected on L-agar containing ampicillin (50 µg/ml) and individual clones isolated. The expression of CTLA4 in these strains was induced by the treatment of exponentially growing cultures with IPTG (0.5 mM) overnight. Extracts were prepared from the culture medium after concentration or by release from periplasm. To prepare periplasmic extracts, cells were incubated in 20 % sucrose, 10 mM Tris-HCl pH7.5 for 15 minutes at room temperature, collected by centrifugation, and resuspended in 4 °C water and held on ice for 10 min. Extracts were assayed for the presence of CTLA4 by SDS-PAGE, Western blotting and competitive B7-1 binding ELISA (as described in Example 2). As shown in Figure 8, soluble CTLA4 prepared from periplasmic extracts of *E. coli* or from the media of these cultures was able to compete for binding to B7-1 with unlabelled CTLA4Ig. In contrast, periplasmic extracts from *E. coli* transfected with the vector alone or media from these cultures was not able to compete for binding to B7-1.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

5

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
(A) NAME: REPLIGEN CORPORATION
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10 (D) STATE: MASSACHUSETTS
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(F) POSTAL CODE (ZIP): 02194
- 15 (ii) TITLE OF INVENTION: ANTIBODIES AND IMMUNOGLOBULIN FUSION
PROTEINS HAVING MODIFIED EFFECTOR FUNCTIONS AND USES THEREFOR
- (iii) NUMBER OF SEQUENCES: 32
- 20 (iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: USA
25 (F) ZIP: 02109-1875
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
30 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
35 (A) APPLICATION NUMBER: PCT/US97/
(B) FILING DATE: 03 FEBRUARY 1997
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
40 (A) APPLICATION NUMBER: USSN 08/595,590
(B) FILING DATE: 02 FEBRUARY 1996
- (viii) ATTORNEY/AGENT INFORMATION:
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45 (B) REGISTRATION NUMBER: 36,207
(C) REFERENCE/DOCKET NUMBER:
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617)227-7400
50 (B) TELEFAX: (617)227-5941
- (2) INFORMATION FOR SEQ ID NO:1:
- 55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs

SUBSTITUTE SHEET (RULE 26)

- 60 -

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10 CATTCTAGAA CCTCGACAAG CTTGAGATCA CAGTTCTCTC TAC

43

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

25 CAGCAGGCTG GGCCACGTGC ATTGCGGAGT GGACACCTGT GGAGAG

46

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

40 CTCTCCACAG GTGTCCACTC CGCAATGCAC GTGGCCCAGC CTGCTG

46

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

55 TGTGTGTGGA ATTCTCATTA CTGATCAGAA TCTGGGCACG GTTCTG

46

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(2) INFORMATION FOR SEQ ID NO:5:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15 GCATTTTAAG CTTTTTCCTG ATCAGGAGCC CAAATCTTCT GACAAAAC TC ACACATCTCC 60
ACCGTCTCCA GGTAAGCC 78

(2) INFORMATION FOR SEQ ID NO:6:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO:7:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

45 GAGCATTTTC CTGATCAGGA GTCCAAATAT GGTCCCCCAC CCCATCATCC CCAGGTAAGC 60
CAACCC 66

(2) INFORMATION FOR SEQ ID NO:8:

- 55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

10 GCAGAGGAAT TCGAGCTCGG TACCCGGGGA TCCCCAGTGT GGGGACAGTG GGACCCGCTC 60
TGCCTCCC 68

15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20

25 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

30 GGGTTTGGG GGAAGAGGA AGACTGACGG TGCCCCCTCG GCTTCAGGTG CTGAGGAAG 59

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35

40

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

45 CATCTCTTCC TCAGCACCTG AAGCCGAGGG GGCACCGTCA GTCTTCTCT TCCCCC 56

(2) INFORMATION FOR SEQ ID NO:11:

50

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55

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(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCACGTGAC CTCAGGGGTC CGGGAGATCA TGAGAGTGTC CTTGGGTTTT GGGGGGAACA 60

10 GGAAGACTGA TGGTGCCCCC TCGAACTCAG GTGCTGAGG 99

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 99 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

25

CCTCAGCACC TGAGTTCGAG GGGGCACCAT CAGTCTTCCT GTTCCCCCA AAACCCAAGG 60

ACACTCTCAT GATCTCCCGG ACCCCTGAGG TCACGTGCG 99

30 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

35

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

45 CATTGCTTA CCTCGACAAG CTTGAGATCA CAGTTCTCTC TAC 43

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

50

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: cDNA

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- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
- 5 GGAGTGGACA CCTGTGGAGA G 21
- (2) INFORMATION FOR SEQ ID NO:15:
- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 15 (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
- 20 CTCCACAGGT GTCCACTCCG CAATGCACGT GGCCCAGCC 39
- (2) INFORMATION FOR SEQ ID NO:16:
- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
- 35 GAGGTTGTAA GGACTCACCT GAAATCTGGG CTCCGTTGC 39
- (2) INFORMATION FOR SEQ ID NO:17:
- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 45 (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
- 50 GCAACGGAGC CCAGATTTC GGTGAGTCCT TACAACCTC 39
- 55 (2) INFORMATION FOR SEQ ID NO:18:

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- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
GGCTAGATAT CTCTAGACTA TAAATCTCTG GCCATGAAG
- (2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 73 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
GGCACTAGGT CGACTCTAGA AACTGAGGAA GCAAAGTTTA AATTCTACTC ACGTTTAATC
TGGGCTCCGT TGC
- (2) INFORMATION FOR SEQ ID NO:20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
GCAGAGAGAC ATATGGCAAT GCACGTGGCC CAGCCTGCTG TGG
- (2) INFORMATION FOR SEQ ID NO:21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

10 GCAGAGAGAG GATCCTCAGT CAGTTAGTCA GAATCTGGGC ACGGTTCTGG 50

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 107 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

25 GGCCTAGTC ATGAAATACC TATTGCCTAC GGCAGCCGCT GGATTGTTAT TACTCGCTGC 60

CCAACCAGCG ATGGCCGCAG CAATGCACGT GGCCAGCCT GCTGTGG 107

30

(2) INFORMATION FOR SEQ ID NO:23:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1705 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CATTCGCTTA CCTCGAGAAG CTTGAGATCA CAGTTCTCTC TACAGTTACT GAGCACACAG 60

50 GACCTCACCA TGGGATGGAG CTGTATCATC CTCTTCTTGG TAGCAACAGC TACAGGTAAG 120

GGGCTCACAG TAGCAGGCTT GAGGTCTGGA CATATATATG GGTGACAATG ACATCCACTT 180

TGCCTTTCTC TCCACAGGTG TCCACTCCGC AATGCACGTG GCCCAGCCTG CTGTGGTACT 240

55 GGCCAGCAGC CGAGGCATCG CCAGCTTTGT GTGTGAGTAT GCATCTCCAG GCAAAGCCAC 300

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TGAGGTCCGG GTGACAGTGC TTCGGCAGGC TGACAGCCAG GTGACTGAAG TCTGTGCGGC 360
 AACCTACATG ATGGGGAATG AGTTGACCTT CCTAGATGAT TCCATCTGCA CGGGCACCTC 420
 5 CAGTGGAAT CAAGTGAACC TCACTATCCA AGGACTGAGG GCCATGGACA CGGGACTCTA 480
 CATCTGCAAG GTGGAGCTCA TGTACCCACC GCCATACTAC CTGGGCATAG GCAACGGAAC 540
 10 CCAGATTTAT GTAATTGATC CAGAACCGTG CCCAGATTCT GATCAGGAGC CCAATCTTC 600
 TGACAAAAC CACACATCTC CACCGTCTCC AGGTAAGCCA GCCCAGGCCT CGCCCTCCAG 660
 CTCAAGGCGG GACAGGTGCC CTAGAGTAGC CTGCATCCAG GGACAGGCC CAGCCGGGTG 720
 15 CTGACACGTC CACCTCCATC TCTTCTCAG CACCTGAAGC CGAGGGGGCA CCGTCAGTCT 780
 TCCTCTTCCC CCCAAAACCC AAGGACACCC TCATGATCTC CCGGACCCCT GAGGTCACAT 840
 20 GCGTGGTGGT GGACGTGAGC CACGAAGACC CTGAGGTCAA GTTCAACTGG TACGTGGACG 900
 GCGTGGAGGT GCATAATGCC AAGACAAAGC CGCGGGAGGA GCAGTACAAC AGCACGTACC 960
 GGGTGGTCAG CGTCCTCACC GTCCTGCACC AGGACTGGCT GAATGGCAAG GAGTACAAGT 1020
 25 GCAAGGTCTC CAACAAAGCC CTCCCAGCCC CCATCGAGAA AACCATCTCC AAAGCCAAAG 1080
 GTGGGACCCG TGGGGTGC GAAGCCACATG GACAGAGGCC GGCTCGGCC ACCCTCTGCC 1140
 30 CTGAGAGTGA CCGCTGTACC AACCTCTGTC CTACAGGGCA GCGCCGAGAA CCACAGGTGT 1200
 ACACCCTGCC CCCATCCCGG GATGAGCTGA CCAAGAACCA GGTCAGCCTG ACCTGCCTGG 1260
 TCAAAGGCTT CTATCCAGC GACATCGCCC TGGAGTGGGA GAGCAATGGG CAGCCGGAGA 1320
 35 ACAACTACAA GACCACGCCT CCCGTGCTGG ACTCCGACGG CTCTTCTTC CTCTACAGCA 1380
 AGCTCACCGT GGACAAGAGC AGGTGGCAGC AGGGGAACGT CTTCTCATGC TCCGTGATGC 1440
 40 ATGAGGCTCT GCACAACCAC TACACGCAGA AGAGCCTCTC CCTGTCTCCG GTTAAATGAG 1500
 TCGACGCGC GGCAAGCCCC GCTCCCGGG CTCTCGCGGT CGCACGAGGA TGCTTGGCAC 1560
 GTACCCCTG TACATACTC CCGGGCGCCC AGCATGAAA TAAAGCACCC AGCGCTGCCC 1620
 45 TGGGCCCCTG CGAGACTGTG ATGGTTCTTT CCACGGGTCA GGCCGAGTCT GAGGCCTGAG 1680
 TGGCATGAGG GAGGCAGAGC GGGTC 1705

50 (2) INFORMATION FOR SEQ ID NO:24:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 377 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 55

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(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

10 Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
 1 5 10 15
 Val His Ser Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala Ser
 20 25 30
 15 Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly Lys
 35 40 45
 Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln Val
 50 55 60
 20 Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr Phe
 65 70 75 80
 25 Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val Asn
 85 90 95
 Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile Cys
 100 105 110
 30 Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly Asn
 115 120 125
 Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser Asp
 130 135 140
 35 Gln Glu Pro Lys Ser Ser Asp Lys Thr His Thr Ser Pro Pro Ser Pro
 145 150 155 160
 40 Ala Pro Glu Ala Glu Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys
 165 170 175
 Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
 180 185 190
 45 Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
 195 200 205
 Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
 210 215 220
 50 Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
 225 230 235 240
 55 Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
 245 250 255

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[illegible]

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1747 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

40	CATTCGCTTA CCTCGAGAAG CTTGAGATCA CAGTTCTCTC TACAGTTACT GAGCACACAG	60
	GACCTCACCA TGGGATGGAG CTGTATCATC CTCTTCTTGG TAGCAACAGC TACAGGTAAG	120
45	GGGCTCACAG TAGCAGGCTT GAGGTCTGGA CATATATATG GGTGACAATG ACATCCACTT	180
	TGCCTTTCTC TCCACAGGTG TCCACTCCGC AATGCACGTG GCCCAGCCTG CTGTGGTACT	240
	GGCCAGCAGC CGAGGCATCG CCAGCTTTGT GTGTGAGTAT GCATCTCCAG GCAAAGCCAC	300
50	TGAGGTCCGG GTGACAGTGC TTCGGCAGGC TGACAGCCAG GTGACTGAAG TCTGTGCGGC	360
	AACCTACATG ATGGGGAATG AGTTGACCTT CCTAGATGAT TCCATCTGCA CGGGCACCTC	420
55	CAGTGGAAT CAAGTGAACC TCACTATCCA AGGACTGAGG GCCATGGACA CGGGACTCTA	480

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CATCTGCAAG GTGGAGCTCA TGTACCCACC GCCATACTAC CTGGGCATAG GCAACGGAAC 540
CCAGATTTAT GTAATTGATC CAGAACCGTG CCCAGATTCT GATCAGGAGT CCAAATATGG 600
5 TCCCCATCC CCATCATCCC CAGGTAAGCC AACCCAGGCC TCGCCCTCCA GCTCAAGGCG 660
GGACAGGTGC CCTAGAGTAG CCTGCATCCA GGGACAGGCC CCAGCCGGGT GCTGACGCAT 720
10 CCACCTCCAT CTCTTCCTCA GCACCTGAGT TCCTGGGGGG ACCATCAGTC TTCCTGTTCC 780
CCCCAAAACC CAAGGACACT CTCATGATCT CCCGGACCCC TGAGGTCACG TCGTGGTGG 840
TGGACGTGAG CCAGGAAGAC CCCGAGGTCC AGTTCAACTG GTACGTGGAT GCGTGGAGG 900
15 TGCATAATGC CAAGACAAAG CCGCGGGAGG AGCAGTTCAA CAGCACGTAC CGTGTGGTCA 960
GCGTCCTCAC CGTCCTGCAC CAGGACTGGC TGAACGGCAA GGAGTACAAG TGCAAGGTCT 1020
CCAACAAAGG CCTCCCGTCC TCCATCGAGA AAACCATCTC CAAAGCCAAA GGTGGGACCC 1080
20 ACGGGGTGCG AGGGCCACAC GGACAGAGGC CAGCTCGGCC CACCCTCTGC CCTGGGAGTG 1140
ACCGCTGTGC CAACCTCTGT CCCTACAGGG CAGCCCCGAG AGCCACAGGT GTACACCCTG 1200
25 CCCCCATCCC AGGAGGAGAT GACCAAGAAC CAGGTCAGCC TGACCTGCCT GGTCAAAGGC 1260
TTCTACCCCA GCGACATCGC CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC 1320
AAGACCACGC CTCCCGTGCT GGACTCCGAC GGCTCCTTCT TCCTCTACAG CAGGCTAACC 1380
30 GTGGACAAGA GCAGGTGGCA GGAGGGGAAT GTCTTCTCAT GCTCCGTGAT GCATGAGGCT 1440
CTGCACAACC ACTACACACA GAAGAGCCTC TCCCTGTCTC TGGGTAAATG AGTGCCAGGG 1500
35 CCGGCAAGCC CCCGCTCCCC GGGCTCTCGG GGTCGCGCGA GGATGCTTGG CACGTACCCC 1560
GTCTACATAC TTCCCAGGCA CCCAGCATGG AAATAAAGCA CCCACCACTG CCCTGGGCCC 1620
CTGTGAGACT GTGATGGTTC TTTCCACGGG TCAGGCCGAG TCTGAGGCCT GAGTGACATG 1680
40 AGGGAGGCAG AGCGGTCCCA CTGTCCCCAC ACTGGGGATC CCCGGGTACC GAGCTCGATT 1740
CCTCTGC 1747

45 (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 374 amino acids

(B) TYPE: amino acid

50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

55

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

5	Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly	1	5	10	15
	Val His Ser Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala Ser	20	25	30	
10	Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly Lys	35	40	45	
	Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln Val	50	55	60	
15	Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr Phe	65	70	75	80
	Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val Asn	85	90	95	
20	Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile Cys	100	105	110	
25	Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly Asn	115	120	125	
	Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser Asp	130	135	140	
30	Gln Glu Ser Lys Tyr Gly Pro Pro Ser Pro Ser Ser Pro Ala Pro Glu	145	150	155	160
	Phe Glu Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp	165	170	175	
35	Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp	180	185	190	
40	Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly	195	200	205	
	Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn	210	215	220	
45	Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp	225	230	235	240
	Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro	245	250	255	
50	Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu	260	265	270	
55	Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn				

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	275	280	285
5	Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile 290 295 300		
	Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr 305 310 315 320		
10	Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg 325 330 335		
	Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys 340 345 350		
15	Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu 355 360 365		
20	Ser Leu Ser Leu Gly Lys 370		

(2) INFORMATION FOR SEQ ID NO:27:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1747 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

	CATTGCGCTTA CCTCGAGAAG CTTGAGATCA CAGTTCTCTC TACAGTTACT GAGCACACAG	60
40	GACCTCACCA TGGGATGGAG CTGTATCATC CTCTTCTTGG TAGCAACAGC TACAGGTAAG	120
	GGGCTCACAG TAGCAGGCTT GAGGTCTGGA CATATATATG GGTGACAATG ACATCCACTT	180
	TGCCTTTCTC TCCACAGGTG TCCACTCCGC AATGCACGTG GCCCAGCCTG CTGTGGTACT	240
45	GGCCAGCAGC CGAGGCATCG CCAGCTTTGT GTGTGAGTAT GCATCTCCAG GCAAAGCCAC	300
	TGAGGTCCGG GTGACAGTGC TTCGGCAGGC TGACAGCCAG GTGACTGAAG TCTGTGCGGC	360
50	AACCTACATG ATGGGGAATG AGTTGACCTT CCTAGATGAT TCCATCTGCA CGGGCACCTC	420
	CAGTGGAAAT CAAGTGAACC TCACTATCCA AGGACTGAGG GCCATGGACA CGGGACTCTA	480
	CATCTGCAAG GTGGAGCTCA TGTACCCACC GCCATACTAC CTGGGCATAG GCAACGGAAC	540
55	CCAGATTAT GTAATTGATC CAGAACCGTG CCCAGATTCT GATCAGGAGT CCAAATATGG	600

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TCCCCCATCC CCATCATCCC CAGGTAAGCC AACCCAGGCC TCGCCCTCCA GCTCAAGGCG 660
5 GGACAGGTGC CCTAGAGTAG CCTGCATCCA GGGACAGGCC CCAGCCGGGT GCTGACGCAT 720
CCACCTCCAT CTCTTCCTCA GCACCTGAGT TCGAGGGGGC ACCATCAGTC TTCCTGTTCC 780
CCCCAAAACC CAAGGACACT CTCATGATCT CCCGGACCCC TGAGGTCACG TCGGTGGTGG 840
10 TGGACGTGAG CCAGGAAGAC CCCGAGGTCC AGTTCAACTG GTACGTGGAT GGCCTGGAGG 900
TGCATAATGC CAAGACAAAG CCGCGGGAGG AGCAGTTCAA CAGCACGTAC CGTGTGGTCA 960
15 GCGTCCTCAC CGTCTGCAC CAGGACTGGC TGAACGGCAA GGAGTACAAG TGCAAGGTCT 1020
CCAACAAAGG CCTCCCGTCC TCCATCGAGA AAACCATCTC CAAAGCCAAA GGTGGGACCC 1080
ACGGGGTGCG AGGGCCACAC GGACAGAGGC CAGCTCGGCC CACCCTCTGC CCTGGGAGTG 1140
20 ACCGCTGTGC CAACCTCTGT CCCTACAGGG CAGCCCCGAG AGCCACAGGT GTACACCCTG 1200
CCCCCATCCC AGGAGGAGAT GACCAAGAAC CAGGTCAGCC TGACCTGCCT GGTCAAAGGC 1260
25 TTCTACCCCA GCGACATCGC CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC 1320
AAGACCACGC CTCCCGTGCT GGA CTCCGAC GGCTCCTTCT TCCTCTACAG CAGGCTAACC 1380
GTGGACAAGA GCAGGTGGCA GGAGGGGAAT GTCTTCTCAT GCTCCGTGAT GCATGAGGCT 1440
30 CTGCACAACC ACTACACACA GAAGAGCCTC TCCCTGTCTC TGGGTAAATG AGTGCCAGGG 1500
CCGGCAAGCC CCCGCTCCCC GGGCTCTCGG GGTGCGCGGA GGATGCTTGG CACGTACCCC 1560
35 GTCTACATAC TTCCAGGCA CCCAGCATGG AAATAAAGCA CCCACCACTG CCCTGGGCCC 1620
CTGTGAGACT GTGATGGTTC TTTCCACGGG TCAGGCCGAG TCTGAGGCCT GAGTGACATG 1680
AGGGAGGCAG AGCGGTCCCA CTGTCCCCAC ACTGGGGATC CCCGGGTACC GAGCTCGATT 1740
40 CCTCTGC 1747

45 (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 374 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

55

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

5	Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly	1	5	10	15
	Val His Ser Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala Ser	20	25	30	
10	Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly Lys	35	40	45	
	Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln Val	50	55	60	
15	Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr Phe	65	70	75	80
	Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val Asn	85	90	95	
20	Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile Cys	100	105	110	
	Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly Asn	115	120	125	
	Gly Thr Gln Ile Tyr Val Ile Asp Pro Glu Pro Cys Pro Asp Ser Asp	130	135	140	
30	Gln Glu Ser Lys Tyr Gly Pro Pro Ser Pro Ser Ser Pro Ala Pro Glu	145	150	155	160
	Phe Glu Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp	165	170	175	
35	Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp	180	185	190	
	Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly	195	200	205	
	Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn	210	215	220	
45	Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp	225	230	235	240
	Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro	245	250	255	
50	Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu	260	265	270	
55	Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn				

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275 280 285

Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
290 295 300

5 Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
305 310 315 320

10 Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg
325 330 335

Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys
340 345 350

15 Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
355 360 365

Ser Leu Ser Leu Gly Lys
370

20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2770 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

35 CATTCGCTTA CCTCGAGAAG CTTGAGATCA CAGTTCTCTC TACAGTTACT GAGCACACAG 60

GACCTCACCA TGGGATGGAG CTGTATCATC CTCTTCTTGG TAGCAACAGC TACAGGTAAG 20

GGGCTCACAG TAGCAGGCTT GAGGTCTGGA CATATATATG GGTGACAATG ACATCCACTT 80

40 TGCCTTTCTC TCCACAGGTG TCCACTCCGC AATGCACGTG GCCCAGCCTG CTGTGGTACT 40

GGCCAGCAGC CGAGGCATCG CCAGCTTTGT GTGTGAGTAT GCATCTCCAG GCAAAGCCAC 00

45 TGAGGTCGGG GTGACAGTGC TTCGGCAGGC TGACAGCCAG GTGACTGAAG TCTGTGCGGC 60

AACCTACATG ATGGGGAATG AGTTGACCTT CCTAGATGAT TCCATCTGCA CGGGCACCTC 20

CAGTGGAAAT CAAGTGAACC TCACTATCCA AGGACTGAGG GCCATGGACA CGGGACTCTA 80

50 CATCTGCAAG GTGGAGCTCA TGTACCCACC GCCATACTAC CTGGGCATAG GCAACGGAGC 40

CCAGATTTC A GGTGAGTCCT TACAACCTCT CTCTTCTATT CAGCTTAAAT AGATTTTACT 00

55 GCATTTGTTG GGGGGGAAAT GTGTGTATCT GAATTCAGG TCATGAAGGA CTAGGGACAC 60

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	CTTGGGAGTC AGAAAGGGTC ATTGGGAGCC CGGGCTGATG CAGACAGACA TCCTCAGCTC	20
5	CCAGACTTCA TGGCCAGAGA TTTATAGTCT AGAGGATCCC CAGCTTTCTG GGGCAGGCCA	80
	GGCCTGACCT TGGCTTTGGG GCAGGGAGGG GGCTAAGGTG AGGCAGGTGG CGCCAGCAGG	40
	TGCACACCCA ATGCCCATGA GCCCAGACAC TGGACGCTGA ACCTCGCGGA CAGTTAAGAA	00
10	CCCAGGGGCC TCTGCGCCTG GGCCCAAGCTC TGTCCACAC CGCGGTCACA TGGCACCACC	60
	TCTCTTGACG CCTCCACCAA GGGCCCATCG GTCTTCCCC TGGCACCCCTC CTCCAAGAGC	20
15	ACCTCTGGGG GCACAGCGGC CCTGGGCTGC CTGGTCAAGG ACTACTTCCC CGAACCGGTG	80
	ACGGTGTCTG GGAAGTCAGG CGCCCTGACC AGCGGCGTGC ACACCTTCCC GGCTGTCCTA	40
	CAGTCTCAG GACTCTACTC CCTCAGCAGC GTGGTGACCG TGCCCTCCAG CAGCTTGGGC	00
20	ACCCAGACCT ACATCTGCAA CGTGAATCAC AAGCCCAGCA ACACCAAGGT GGACAAGAAA	60
	GTTGGTGAGA GGCCAGCACA GGGAGGGAGG GTGTCTGCTG GAAGCAGGCT CAGCGCTCCT	20
25	GCCTGGACGC ATCCCGGCTA TGCAGCCCCA GTCCAGGGCA GCAAGGCAGG CCCCCTCTGC	80
	CTCTTCACCC GGAGCCTCTG CCCGCCCCAC TCATGCTCAG GGAGAGGGTC TTCTGGCTTT	40
	TTCCCAGGCT CTGGGCAGGC ACAGGCTAGG TGCCCTAAC CCAGGCCCTG CACACAAAGG	00
30	GGCAGGTGCT GGGCTCAGAC CTGCCAAGAG CCATATCCGG GAGGACCCTG CCCCTGACCT	60
	AAGCCCACCC CAAAGGCCAA ACTCTCCACT CCCTCAGCTC GGACACCTC TCTCCTCCCA	20
35	GATTCCAGTA ACTCCCAATC TTCTCTCTGC AGAGCCCAAA TCTGTGACA AAATCAGAC	80
	ATGCCCACCG TGCCAGGTA AGCCAGCCCA GGCTCGCCC TCCAGCTCAA GGCGGGACAG	40
	GTGCCCTAGA GTAGCCTGCA TCCAGGGACA GGCCCCAGCC GGGTGCTGAC ACGTCCACCT	00
40	CCATCTCTTC CTCAGCACCT GAACTCCTGG GGGGACCGTC AGTCTTCCTC TTCCCCCAA	60
	AACCCAAGGA CACCTCATG ATCTCCCGGA CCCCTGAGGT CACATGCGTG GTGGTGACG	20
45	TGAGCCACGA AGACCCTGAG GTCAAGTTCA ACTGGTACGT GGACGGCGTG GAGGTGCATA	80
	ATGCCAAGAC AAAGCCGCGG GAGGAGCAGT ACAACAGCAC GTACCGGGTG GTCAGCGTCC	040
	TCACCGTCCT GCACCAGGAC TGGCTGAATG GCAAGGAGTA CAAGTGCAAG GTCTCCAACA	100
50	AAGCCCTCCC AGCCCCATC GAGAAAACCA TCTCCAAAGC CAAAGGTGGG ACCCGTGGGG	160
	TGCGAGGGCC ACATGGACAG AGGCCGGCTC GGCCACCCT CTGCCCTGAG AGTGACCGCT	220
55	GTACCAACCT CTGTCTACA GGGCAGCCCC GAGAACCACA GGTGTACACC CTGCCCCCAT	280

- 77 -

CCCGGGATGA GCTGACCAAG AACCAGGTCA GCCTGACCTG CCTGGTCAAA GGCTTCTATC 340
 CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC TACAAGACCA 400
 5 CGCCTCCCGT GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC ACCGTGGACA 460
 AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG GCTCTGCACA 520
 ACCACTACAC GCAGAAGAGC CTCTCCCTGT CTCCGGGTAA ATGAGTGGCA CGGCCGGCAA 580
 10 GCCCCGCTCC CCGGGCTCTC GCGGTGCGAC GAGGATGCTT GGCACGTACC CCCTGTACAT 640
 ACTTCCCGGG CGCCCAGCAT GGAATAAAG CACCCAGCGC TGCCCTGGGC CCCTGCGAGA 700
 15 CTGTGATGGT TCTTTCACG GGTGAGGCCG AGTCTGAGGC CTGAGTGGCA TGAGGGAGGC 760
 AGAGCGGGTC 770

(2) INFORMATION FOR SEQ ID NO:30:
 20

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 238 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

35 Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
 1 5 10 15
 Val His Ser Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala Ser
 20 25 30
 40 Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly Lys
 35 40 45
 Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln Val
 50 55 60
 45 Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr Phe
 65 70 75 80
 50 Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val Asn
 85 90 95
 Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile Cys
 100 105 110
 55 Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly Asn

- 78 -

115 120 125
 Gly Ala Gln Ile Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
 130 135 140
 5 Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu
 145 150 155 160
 10 Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn
 165 170 175
 Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
 180 185 190
 15 Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala
 195 200 205
 20 Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly
 210 215 220
 Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230 235

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1708 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CATTCGCTTA CCTCGAGAAG CTTGAGATCA CAGTTCTCTC TACAGTTACT GAGCACACAG 60
 40 GACCTCACCA TGGGATGGAG CTGTATCATC CTCTTCTTGG TAGCAACAGC TACAGGTAAG 20
 GGGCTCACAG TAGCAGGCTT GAGGTCTGGA CATATATATG GGTGACAATG ACATCCACTT 80
 45 TGCCTTTCTC TCCACAGGTG TCCACTCCGC AATGCACGTG GCCCAGCCTG CTGTGGTACT 40
 GGCCAGCAGC CGAGGCATCG CCAGCTTTGT GTGTGAGTAT GCATCTCCAG GCAAAGCCAC 00
 TGAGGTCCGG GTGACAGTGC TTCGGCAGGC TGACAGCCAG GTGACTGAAG TCTGTGCGGC 60
 50 AACCTACATG ATGGGGAATG AGTTGACCTT CCTAGATGAT TCCATCTGCA CGGGCACCTC 20
 CAGTGGAAT CAAGTGAACC TCACTATCCA AGGACTGAGG GCCATGGACA CGGGACTCTA 80
 55 CATCTGCAAG GTGGAGCTCA TGTACCCACC GCCATACTAC CTGGGCATAG GCAACGGAGC 40

- 79 -

CCAGATTAAA CGTGAGTAGA ATTTAAACTT TGCTTCCTCA GTTCTAGAA GAATGGCTGC 00
 AAAGAGCTCC AACAAACAA TTTAGAACTT TATTAAGGAA TAGGGGGAAG CTAGGAAGAA 60
 5 ACTCAAACA TCAAGATTTT AAATACGCTT CTTGGTCTCC TTGCTATAAT TATCTGGGAT 20
 AAGCATGCTG TTTTCTGTCT GTCCCTAACA TGCCCTGTGA TTATCCGCAA ACAACACACC 80
 CAAGGGCAGA ACTTTGTTAC TTAAACACCA TCCTGTTTGC TTCTTTCCTC AGGAACTGTG 40
 10 GCTGCACCAT CTGTCTTCAT CTTCCCGCCA TCTGATGAGC AGTTGAAATC TGGAAGTACC 00
 TCTGTGTGTG GCCTGCTGAA TAACTTCTAT CCCAGAGAGG CCAAAGTACA GTGGAAGGTG 60
 15 GATAACGCCC TCCAATCGGG TAACTCCCAG GAGAGTGTCA CAGAGCAGGA CAGCAAGGAC 20
 AGCACCTACA GCCTCAGCAG CACCCTGACG CTGAGCAAAG CAGACTACGA GAAACACAAA 80
 GTCTACGCCT GCGAAGTCAC CCATCAGGGC CTGAGCTCGC CCGTCACAAA GAGCTTCAAC 40
 20 AGGGGAGAGT GTTAGAGGGA GAAGTGCCCC CACCTGCTCC TCAGTTCCAG CCTGACCCCC 00
 TCCCATCCTT TGGCCTCTGA CCCTTTTTC ACAGGGGACC TACCCCTATT GCGGTCCTCC 60
 25 AGCTCATCTT TCACCTCACC CCCCTCCTCC TCCTTGGCTT TAATTATGCT AATGTTGGAG 20
 GAGAATGAAT AAATAAAGTG AATCTTTGCA CCTGTGGTTT CTCTCTTTC TCAATTTAAT 80
 AATTATTATC TGTTGTTTAC CAACTACTCA ATTTCTCTTA TAAGGGACTA AATATGTAGT 40
 30 CATCCTAAGG CGCATAACCA TTTATAAAAA TCATCCTTCA TTCTATTTTA CCCTATCATC 00
 CTCTGCAAGA CAGTCCTCCC TCAAACCCAC AAGCCTTCTG TCCTCACAGT CCCCTGGGCC 60
 35 GTGGTAGGAG AGACTTGCTT CTTGTTTTTC CCCTCCTCAG CAAGCCCTCA TAGTCCTTTT 20
 TAAGGGTGAC AGGTCTTACG GTCATATATC CTTTGATTCA ATTCCCTGGG AATCAACCAA 80
 GGCAAATTTT TCAAAAGAAG AAACCTGC 08
 40

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 238 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (v) FRAGMENT TYPE: internal

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

- 80 -

	Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly	
	1 5 10 15	
5	Val His Ser Ala Met His Val Ala Gln Pro Ala Val Val Leu Ala Ser	
	20 25 30	
	Ser Arg Gly Ile Ala Ser Phe Val Cys Glu Tyr Ala Ser Pro Gly Lys	
	35 40 45	
10	Ala Thr Glu Val Arg Val Thr Val Leu Arg Gln Ala Asp Ser Gln Val	
	50 55 60	
	Thr Glu Val Cys Ala Ala Thr Tyr Met Met Gly Asn Glu Leu Thr Phe	
15	65 70 75 80	
	Leu Asp Asp Ser Ile Cys Thr Gly Thr Ser Ser Gly Asn Gln Val Asn	
	85 90 95	
20	Leu Thr Ile Gln Gly Leu Arg Ala Met Asp Thr Gly Leu Tyr Ile Cys	
	100 105 110	
	Lys Val Glu Leu Met Tyr Pro Pro Pro Tyr Tyr Leu Gly Ile Gly Asn	
	115 120 125	
25	Gly Ala Gln Ile Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro	
	130 135 140	
	Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu	
30	145 150 155 160	
	Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn	
	165 170 175	
35	Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser	
	180 185 190	
	Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala	
	195 200 205	
40	Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly	
	210 215 220	
	Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys	
45	225 230 235	

CLAIMS

1. An isolated nucleic acid encoding a CTLA4-immunoglobulin fusion
5 protein, the nucleic acid comprising a nucleotide sequence encoding a first peptide
having a CTLA4 activity and a nucleotide sequence encoding a second peptide
comprising an immunoglobulin constant region which is modified to reduce at least one
constant region-mediated biological effector function.
- 10 2. An isolated nucleic acid of claim 1, wherein the first peptide comprises
an extracellular domain of the CTLA4 protein.
3. An isolated nucleic acid of claim 2, wherein the first peptide comprises
15 amino acid residues 1-125 of the human CTLA4 protein.
4. An isolated nucleic acid of claim 2, wherein the first peptide binds B7-1
or B7-2.
5. An isolated nucleic acid of claim 1, wherein the immunoglobulin
20 constant region comprises a hinge region, a CH2 domain and a CH3 domain.
6. An isolated nucleic acid of claim 5, wherein the hinge region, the CH2
domain and the CH3 domain are selected from the group consisting of C γ 1, C γ 2, C γ 3
and C γ 4.
25
7. An isolated nucleic acid encoding a CTLA4-immunoglobulin fusion
protein, the nucleic acid comprising a nucleotide sequence encoding a first peptide
having a CTLA4 activity and a nucleotide sequence encoding a second peptide
comprising an immunoglobulin constant region wherein the immunoglobulin constant
30 region comprises a heavy chain CH1 domain, a hinge region, a CH2 domain and a CH3
domain.
8. The isolated nucleic acid of claim 7, wherein the immunoglobulin
constant region is modified to reduce at least one constant region-mediated biological
35 effector function.

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9. An isolated nucleic acid of claim 7, wherein the first peptide having a CTLA4 activity and the hinge region of the second peptide include at least one cysteine residue available for disulfide bond formation.

5 10. The isolated nucleic acid of claim 8, wherein the first peptide having a CTLA4 activity and the hinge region of the second peptide include at least one cysteine residue available for disulfide bond formation.

10 11. An isolated nucleic acid of claim 5, wherein the biological effector function is selected from the group consisting of complement activation, Fc receptor interaction, and complement activation and Fc receptor interaction.

15 12. An isolated nucleic acid of claim 11, wherein at least one amino acid residue selected from a hinge link region of the CH2 domain is modified by substitution, addition or deletion.

20 13. An isolated nucleic acid of claim 12, wherein the at least one amino acid residue of the hinge link region of the CH2 domain is located at a position of a full-length intact immunoglobulin heavy chain selected from the group consisting of position 234, position 235 and position 237.

14. An isolated nucleic acid of claim 13, wherein the CH2 domain is derived from C γ 1.

25 15. An isolated nucleic acid of claim 14, wherein the at least one amino acid residue selected from a hinge link region of the CH2 domain is modified by at least one substitution selected from the group consisting of: substitution of Leu at position 234 with Ala; substitution of Leu at position 235 with Glu; and substitution of Gly at position 237 with Ala.

30 16. An isolated nucleic acid of claim 15, wherein Leu at position 234 is substituted with Ala, Leu at position 235 is substituted with Glu and Gly at position 237 is substituted with Ala.

35 17. An isolated nucleic acid of claim 13, wherein the CH2 domain is derived from C γ 4.

18. An isolated nucleic acid of claim 17, wherein the at least one amino acid residue selected from a hinge link region of the CH2 domain is modified by at least one substitution selected from the group consisting of: substitution of Leu at position 234
5 with Ala; substitution of Leu at position 235 with Glu; and substitution of Gly at position 237 with Ala.

19. An isolated nucleic acid of claim 18, wherein Leu at position 235 is substituted with Glu and Gly at position 237 is substituted with Ala.
10

20. An isolated nucleic acid of claim 11, wherein at least one amino acid residue selected from a hinge-proximal bend region of the CH2 domain is modified by substitution, addition or deletion.

21. An isolated nucleic acid of claim 20, wherein an amino acid residue at position 331 of an intact immunoglobulin heavy chain is modified by substitution with another amino acid residue.
15

22. An isolated nucleic acid of claim 21, wherein the CH2 domain is derived from Cy1 Cy2, Cy3, or Cy4.
20

23. An isolated nucleic acid of claim 21, wherein Pro at position 331 of an intact immunoglobulin heavy chain is substituted with Ser.

24. An isolated nucleic acid of claim 11, wherein at least one amino acid residue of the CH2 domain located at a position of an intact immunoglobulin heavy chain selected from the group consisting of position 318, position 320 and position 322 is modified by substitution, addition or deletion.
25

25. An isolated nucleic acid of claim 24, wherein the at least one amino acid residue of the CH2 domain is modified by at least one substitution selected from the group consisting of: substitution of Glu at position 318 with Ala or Val; substitution of Lys at position 320 with Ala or Gln; and substitution of Lys at position 322 with Ala or Gln.
30

35

26. An isolated nucleic acid of claim 25, wherein Glu at position 318 is substituted with Ala or Val, Lys at position 320 is substituted with Ala or Gln and Lys at position 322 is substituted with Ala or Gln.

5 27. An isolated nucleic acid of claim 5, wherein the hinge region is modified to reduce at least one biological effector function.

28. An isolated nucleic acid of claim 27, wherein the biological effector function is complement activation.

10

29. An isolated nucleic acid of claim 28, wherein at least one amino acid residue located in the hinge region is modified by substitution, addition or deletion.

30. An isolated nucleic acid of claim 29, wherein the immunoglobulin
15 constant region is C γ 1 C γ 2, C γ 3, or C γ 4.

31. An isolated nucleic acid of claim 30, wherein the hinge region of C γ 1 or C γ 3 is substituted with a hinge region derived from C γ 4.

20 32. An isolated nucleic acid of claim 5, wherein the CTLA4-immunoglobulin fusion protein comprises an amino acid sequence shown in SEQ ID NO: 26.

33. An isolated nucleic acid of claim 5 comprising a nucleotide sequence shown in SEQ ID NO: 25.

25

34. An isolated nucleic acid of claim 5, wherein the CH2 domain is modified by substitution of Glu for Leu at position 235 of an intact immunoglobulin heavy chain and by substitution of Ala for Gly at position 237 of an intact immunoglobulin heavy chain.

30

35. An isolated nucleic acid of claim 34, wherein the CTLA4-immunoglobulin fusion protein comprises an amino acid sequence shown in SEQ ID NO: 28.

35 36. An isolated nucleic acid of claim 34 comprising a nucleotide sequence shown in SEQ ID NO: 27.

37. An isolated nucleic acid of claim 5, wherein the CTLA4-immunoglobulin fusion protein comprises an amino acid sequence shown in SEQ ID NO: 24.

5 38. An isolated nucleic acid of claim 5 comprising a nucleotide sequence shown in SEQ ID NO: 23.

39. An isolated nucleic acid encoding a CTLA4-immunoglobulin light chain fusion protein, wherein the nucleic acid comprises a nucleotide sequence encoding a
10 first peptide comprising a CTLA4 extracellular domain and a nucleotide sequence encoding a second peptide comprising an immunoglobulin light chain constant domain.

40. An isolated nucleic acid capable of expression in a bacterial host cell, the nucleic acid consisting of a nucleotide sequence encoding a CTLA4 extracellular
15 domain.

41. An isolated nucleic acid comprising a nucleotide sequence encoding a soluble CTLA4 protein capable of expression in a bacterial host cell, wherein the nucleic acid consists of a nucleotide sequence encoding a signal sequence and a
20 nucleotide sequence encoding a CTLA4 extracellular domain.

42. A recombinant expression vector comprising a nucleic acid of claim 1.

25 43. A recombinant expression vector comprising a nucleic acid of claim 3.

44. A recombinant expression vector comprising a nucleic acid of claim 7.

45. A recombinant expression vector comprising a nucleic acid of claim 8.

30 46. A recombinant expression vector comprising a nucleic acid of claim 39.

47. A recombinant expression vector comprising a nucleic acid of claim 40.

35 48. A recombinant expression vector comprising a nucleic acid of claim 41.

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49. A host cell transfected with the expression vector of claim 38 capable of directing the expression of a CTLA4-immunoglobulin fusion protein.

50. A host cell transfected with the expression vector of claim 43 capable of directing the expression of a CTLA4-immunoglobulin fusion protein.

51. A host cell transfected with the expression vector of claim 7 capable of directing the expression of a CTLA4-immunoglobulin fusion protein.

52. A host cell transfected with the expression vector of claim 8 capable of directing the expression of a CTLA4-immunoglobulin fusion protein.

53. A host cell transfected with the expression vector of claim 46 capable of directing the expression of a CTLA4-immunoglobulin fusion protein.

54. A bacterial host cell transfected with the expression vector of claim 47 capable of directing the expression of a CTLA4 extracellular domain.

55. A bacterial host cell transfected with the expression vector of claim 48 capable of directing the expression of a CTLA4 extracellular domain.

56. A CTLA4-immunoglobulin fusion protein comprising a first peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region which is modified to reduce at least one constant region-mediated biological effector function relative to a CTLA4-IgG1 fusion protein.

57. A CTLA4-immunoglobulin fusion protein of claim 56, wherein the first peptide comprises an extracellular domain of the CTLA4 protein.

58. A CTLA4-immunoglobulin fusion protein of claim 57, wherein the first peptide comprises amino acid residues 1-125 of the human CTLA4 protein.

59. A CTLA4-immunoglobulin fusion protein of claim 56, wherein the immunoglobulin constant region comprises a hinge region, a CH2 domain and a CH3 domain.

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60. A CTLA4-immunoglobulin fusion protein of claim 59, wherein the hinge region, the CH2 domain and the CH3 domain are selected from the group consisting of Cy1, Cy2, Cy3 and Cy4.
- 5 61. A CTLA4-immunoglobulin fusion protein, comprising a first peptide having a CTLA4 activity and a second peptide comprising an immunoglobulin constant region wherein the immunoglobulin constant region comprises a heavy chain CH1 domain, a hinge region, a CH2 domain and a CH3 domain.
- 10 62. The peptide of claim 61, wherein the immunoglobulin constant region is modified to reduce at least one constant region-mediated biological effector function.
- 15 63. The peptide of claim 61, wherein the first peptide having a CTLA4 activity and the hinge region of the second peptide include at least one cysteine residue available for disulfide bond formation.
- 20 64. The isolated nucleic acid of claim 62, wherein the first peptide having a CTLA4 activity and the hinge region of the second peptide include at least one cysteine residue available for disulfide bond formation.
- 25 65. A CTLA4-immunoglobulin fusion protein of claim 59, wherein the CH2 domain is modified to reduce biological effector functions.
- 30 66. A CTLA4-immunoglobulin fusion protein of claim 65, wherein the biological effector function is selected from the group consisting of complement activation, Fc receptor interaction, and complement activation and Fc receptor interaction.
- 35 67. A CTLA4-immunoglobulin fusion protein of claim 66, wherein the CH2 domain is modified by substitution of an amino acid residue located at a position of an intact immunoglobulin heavy chain selected from the group consisting of position 234, position 235 and position 237.
68. A CTLA4-immunoglobulin fusion protein of claim 67 comprising an amino acid sequence shown in SEQ ID NO: 24.

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69. A CTLA4-immunoglobulin fusion protein of claim 68 comprising an amino acid sequence shown in SEQ ID NO: 28.

5 70. A CTLA4-immunoglobulin light chain fusion protein, wherein the first peptide comprises a CTLA4 extracellular domain and the second peptide comprises an immunoglobulin kappa light chain constant domain.

10 71. An isolated peptide consisting of a CTLA4 extracellular domain produced by a bacterial host cell of claim 54.

72. An isolated peptide consisting of a signal sequence and a CTLA4 extracellular domain produced by a bacterial host cell of claim 55.

15 73. A composition suitable for pharmaceutical administration comprising a CTLA4-immunoglobulin fusion protein of claim 56, and a pharmaceutically acceptable carrier.

20 74. A composition suitable for pharmaceutical administration comprising a CTLA4-immunoglobulin fusion protein of claim 58, and a pharmaceutically acceptable carrier.

25 75. A method for producing a CTLA4-immunoglobulin fusion protein, comprising culturing a host cell of claim 49 in a medium to express the protein and isolating the protein from the medium.

76. A method for producing a CTLA4-immunoglobulin fusion protein, comprising culturing a host cell of claim 50 in a medium to express the protein and isolating the protein from the medium.

30 77. A method for producing a CTLA4-immunoglobulin fusion protein, comprising culturing a host cell of claim 54 in a medium to express the protein and purifying the protein from inclusion bodies.

35 78. A method for producing a CTLA4-immunoglobulin fusion protein, comprising culturing a host cell of claim 55 in a medium to express the protein and purifying the protein by release from periplasm.

79. A method for inhibiting an interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell comprising contacting the antigen presenting cell with a CTLA4-immunoglobulin fusion protein of claim 56.

5

80. A method for inhibiting an interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell comprising contacting the antigen presenting cell with a CTLA4-immunoglobulin fusion protein of claim 58.

10

81. A method for treating an autoimmune disease in a subject mediated by interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell, comprising administering to the subject a CTLA4-immunoglobulin fusion protein of claim 56.

15

82. A method for treating an autoimmune disease in a subject mediated by interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell, comprising administering to the subject a CTLA4-immunoglobulin fusion protein of claim 62.

20

83. A method for treating an autoimmune disease in a subject mediated by interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell, comprising administering to the subject a CTLA4-immunoglobulin fusion protein of claim 70.

25

84. A method for treating an autoimmune disease in a subject mediated by interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell, comprising administering to the subject a CTLA4-immunoglobulin fusion protein of claim 71.

30

85. A method for treating an autoimmune disease in a subject mediated by interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell, comprising administering to the subject a CTLA4-immunoglobulin fusion protein of claim 72.

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86. A method of claim 81, wherein the autoimmune disease is selected from the group consisting of diabetes mellitus, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, and autoimmune thyroiditis.

5 87. A method for treating allergy in a subject mediated by interaction of a CTLA4 ligand on an antigen presenting cell with a receptor for the CTLA4 ligand on a T cell, comprising administering to the subject a CTLA4-immunoglobulin fusion protein of claim 56.

10 88. A method for inhibiting graft-versus-host disease (GVHD) in a bone marrow transplant recipient, comprising administering to the recipient a CTLA4-immunoglobulin fusion protein of claim 56.

15 89. A method of claim 88, wherein donor bone marrow is contacted with the CTLA4-immunoglobulin fusion protein and with cells from the transplant recipient *ex vivo* prior to transplantation of the donor bone marrow into the recipient.

20 90. A method for inhibiting rejection of transplanted cells in a transplant recipient, comprising administering to the recipient a CTLA4-immunoglobulin fusion protein of claim 56.

91. A method for identifying molecules which inhibit binding of CTLA4 to a CTLA4 ligand, comprising

25 a) contacting the CTLA4-immunoglobulin fusion protein of claim 56 with:

- i) a CTLA4 ligand, and
- ii) a molecule to be tested,

wherein either the CTLA4-immunoglobulin fusion protein or the CTLA4 ligand is labeled with a detectable substance;

30 b) removing either unbound CTLA4-immunoglobulin fusion protein or unbound CTLA4 ligand; and

c) determining the amount of CTLA4-immunoglobulin fusion protein bound to the CTLA4 ligand,

35 wherein a reduction in the amount of CTLA4-immunoglobulin fusion protein bound to the CTLA4 ligand in the presence of the molecule indicates that the molecule inhibits binding of CTLA4 to the CTLA4 ligand.

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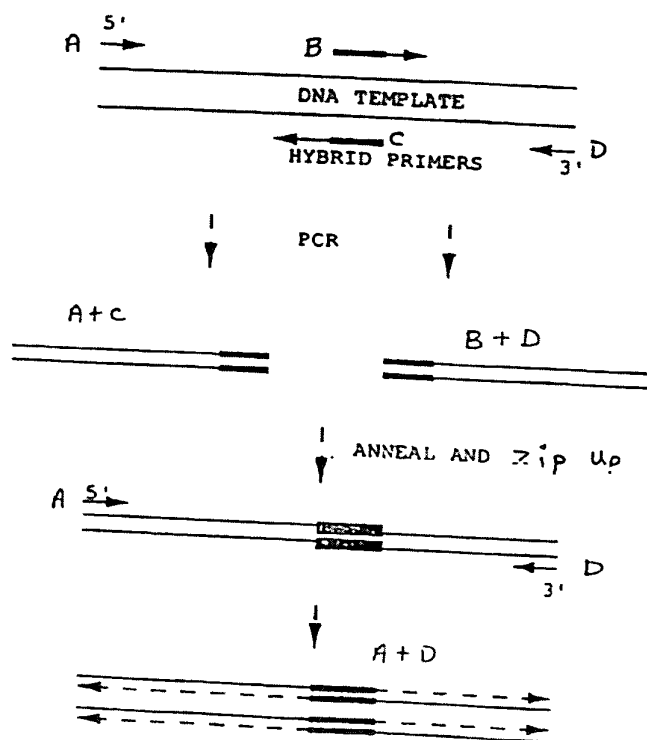


FIGURE 1

A

Gamma 1:

hinge Ch₂

Native A E P K S C D K T H T C P P C P A P E L L G G P S
234 235 237

Mutated A E P K S S D K T H T S P P S P A P E A F G A P S
234 235 237

B Gamma 4:

hinge.....Ch₂.....

Native A E S K Y G P P C P S C P.....A P E F L G G P S.....
234 235 237

Mutated A E S K Y G P P S P S S P.....A P E F G G A P S.....
234 235 237

FIGURE 2

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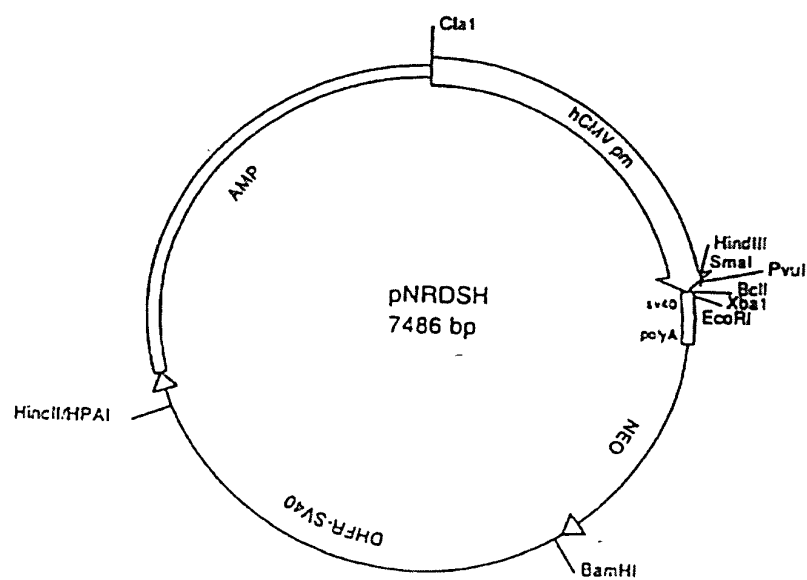


FIGURE 3

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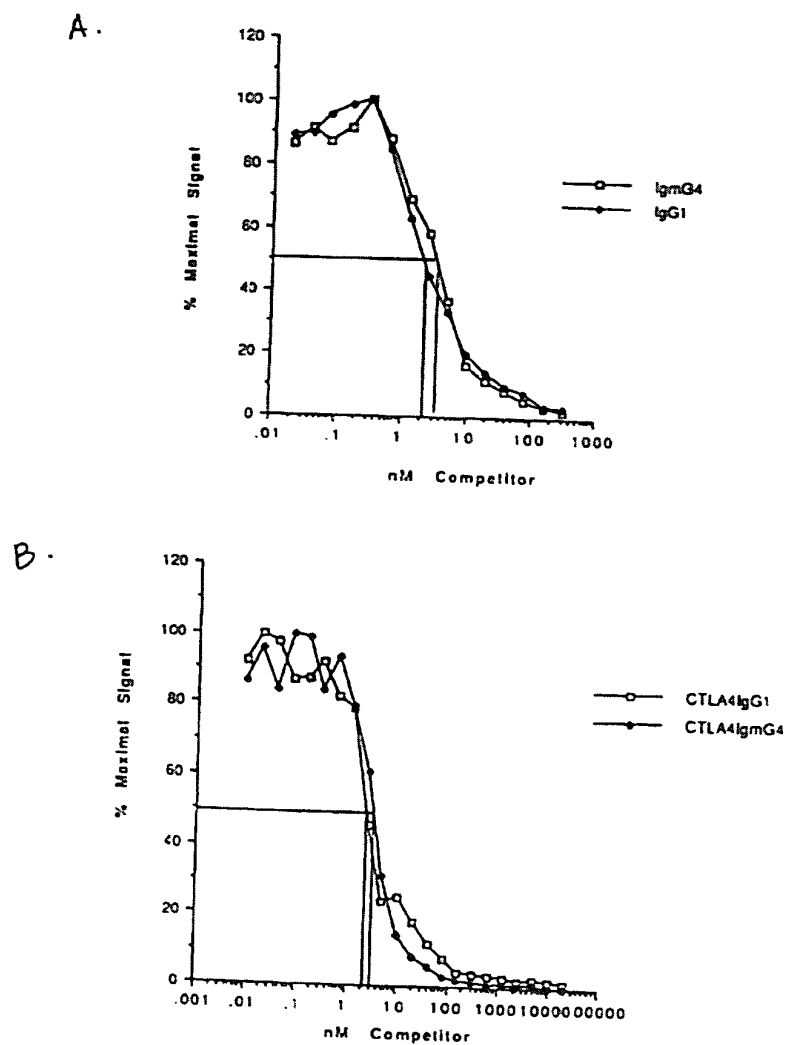


FIGURE 4

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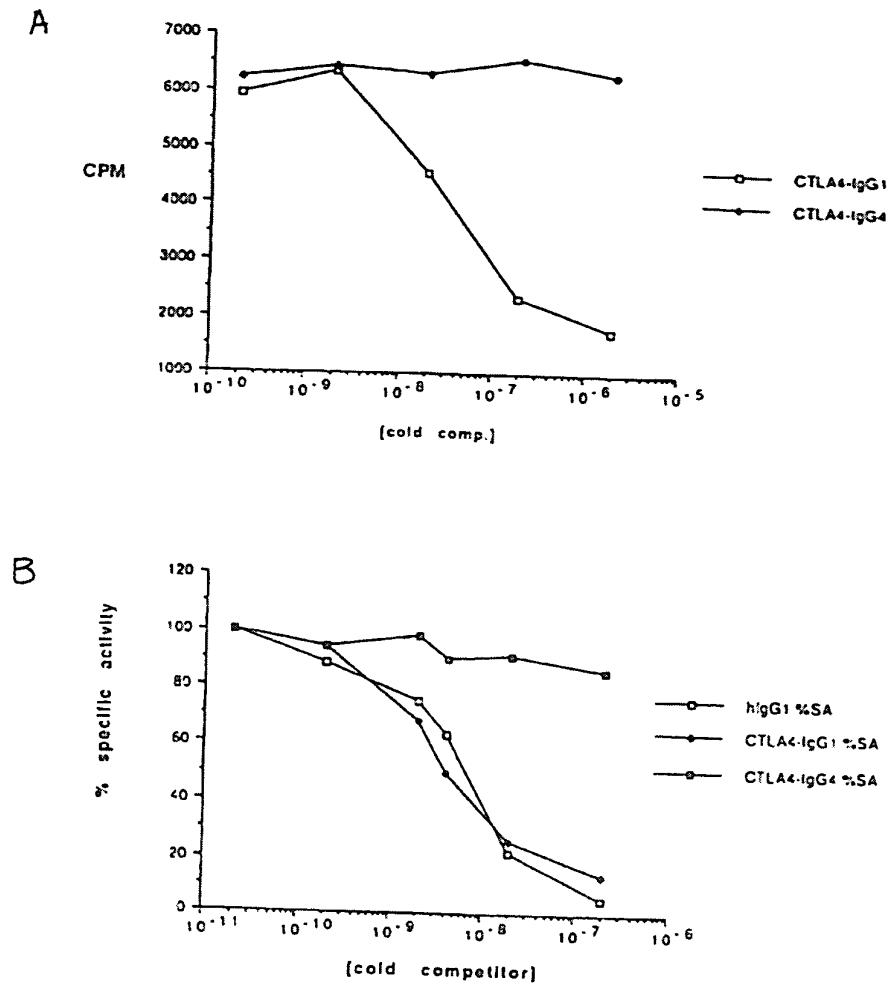


FIGURE 5

G/8

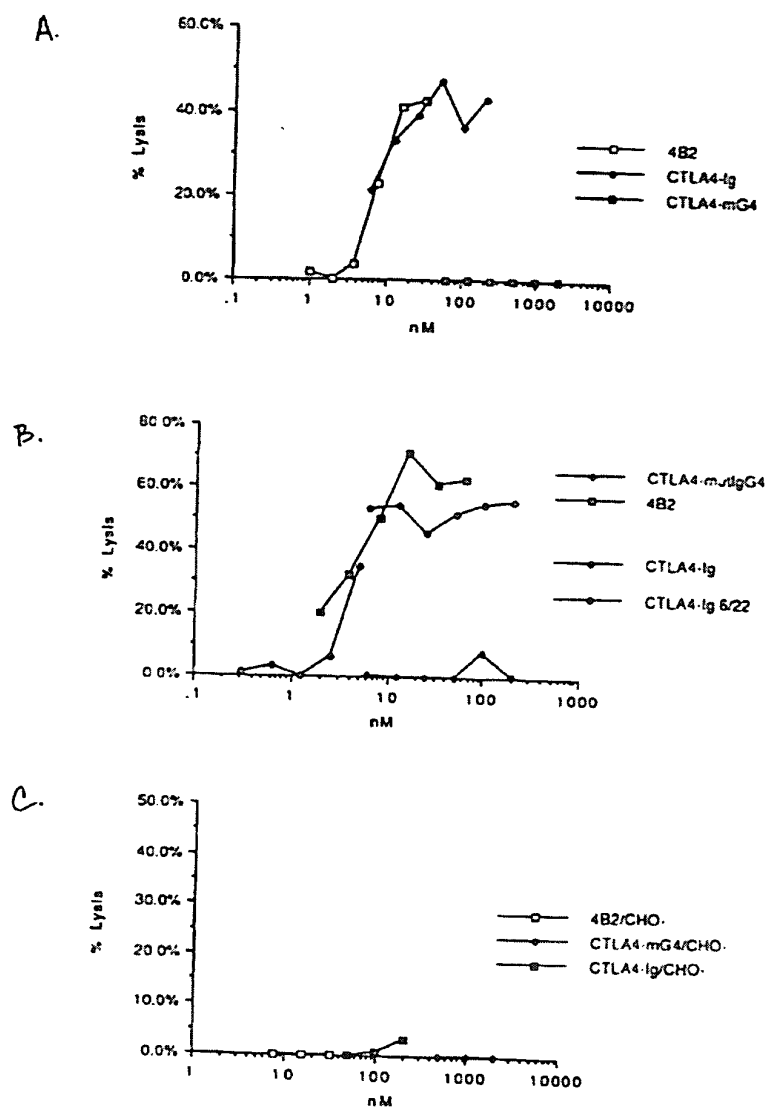


FIGURE 6

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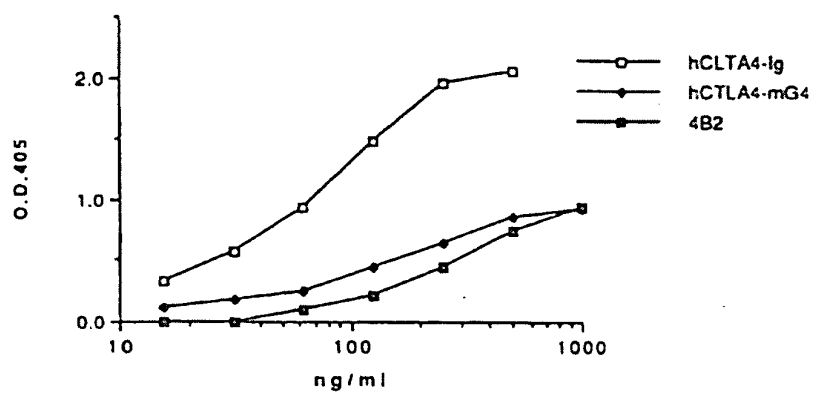
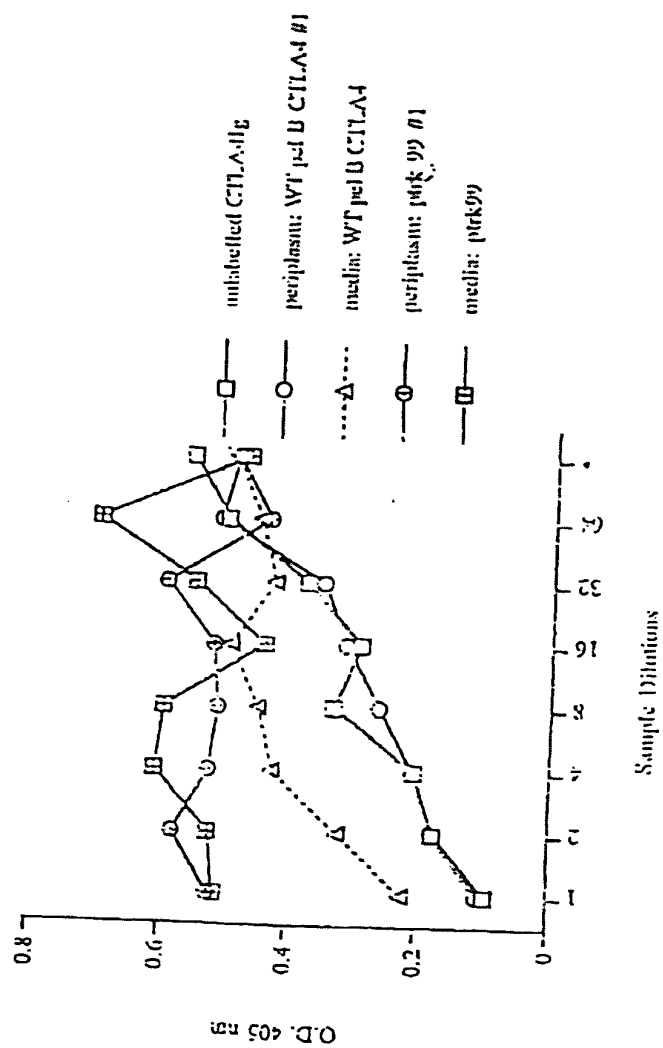


FIGURE 7

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FIGURE 8



INTERNATIONAL SEARCH REPORT

Int'l Application No.

PCT/US 97/01698

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C12N15/12 C12N15/85 C12N15/70 C12N5/10
C12N1/21 C07K19/00 C07K14/705 A61K38/17 G01N33/53
//C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 33770 A (REPLIGEN CORPORATION ET AL.) 14 December 1995 see example 6 --- -/--	40,41, 47,48, 54,55, 71,72

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *A* document member of the same patent family

Date of the actual completion of the international search

16 May 1997

Date of mailing of the international search report

11.06.97

Name and mailing address of the ISA

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Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No
PC1/US 97/01698

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF IMMUNOLOGY, vol. 155, no. 3, 1 August 1995, BALTIMORE, MD, USA, pages 1165-1174, XP002031135 W. STEURER ET AL.: "Ex vivo coating of islet cell allografts with murine CTLA4/Fc promotes graft tolerance."	1-6, 11-13, 20, 24-30, 42,43, 50, 56-60, 65-67, 73,74, 76,79, 80,90,91
Y	see the whole document	
Y	--- SCIENCE, vol. 265, no. 5176, 26 August 1994, WASHINGTON, DC, USA, pages 1225-1227, XP002031136 B. FINCK ET AL.: "Treatment of murine lupus with CTLA4Ig." see abstract	81,86, 88,89 81,86
Y	--- THE JOURNAL OF IMMUNOLOGY, vol. 155, no. 4, 15 August 1995, BALTIMORE, MD, USA, pages 1757-1766, XP002031137 F. HAKIM ET AL.: "Acute graft-versus-host reaction can be aborted by blockade of costimulatory molecules." see abstract	88,89
A	--- NATURE, vol. 332, no. 6166, 21 April 1988, LONDON, GB, pages 738-740, XP002031138 A. DUNCAN ET AL.: "The binding site for C1q on IgG." cited in the application see abstract	1-91
A	--- THE JOURNAL OF IMMUNOLOGY, vol. 147, no. 8, 15 October 1991, BALTIMORE, MD, USA, pages 2657-2662, XP002031139 J. LUND ET AL.: "Human FcgammaRI and FcgammaRII interact with distinct but overlapping sites on human IgG." cited in the application see abstract	1-91
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INTERNATIONAL SEARCH REPORT

International Application No.

PC1/US 97/01698

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>WO 96 31229 A (BETH ISRAEL HOSPITAL ASSOCIATION) 10 October 1996</p> <p>see the whole document -----</p>	<p>1-6, 11-13, 20, 24-30, 42,43, 50, 56-60, 65-67, 73,74, 76,79, 80,90,91</p>

Form PCT/ISA/218 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/01698

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 79,80,81-90
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 79 and 80 (both partially as far as in vivo method is concerned) and 81 to 90 (all completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/01698

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9533770 A	14-12-95	AU 2692095 A CA 2191610 A EP 0764171 A	04-01-96 14-12-95 26-03-97
WO 9631229 A	10-10-96	NONE	